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THE FORMATION OF METHEMOGLOBIN BY STREPTOCOCCUS VIRIDANS.

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(Received for publication, May 29, 1916.)

Certain strains of streptococci, when grown on blood agar plates, produce a green color about the colonies, a characteristic which serves to differentiate them from other strains which produce a clear hemolytic zone. It has long been recognized that the change produced in the blood by these streptococci is a transformation of oxyhemoglobin to methemoglobin, and it is this metabolic function which serves to identify them as belonging to the type now generally called *Streptococcus viridans*.

A similar transformation of oxyhemoglobin to methemoglobin is caused by the pneumococcus. Cole[1] has studied the nature of this reaction and has found that it occurs only when the pneumococci are living and, furthermore, only in the presence of certain nutritive materials, although this material need be present only in very minute quantities. He concludes that the process is due to the alteration of the oxidative processes by the pneumococci in the vicinity of the red blood cells and not to the production of any injurious substance capable of isolation; and therefore that the pathological effects of bacteria on other tissue cells may be due to disturbances in oxidation in the immediate neighborhood of the bacteria and not necessarily to the action of a definite poison.

The part which *Streptococcus viridans* plays in human pathology is becoming increasingly evident and for that reason it has seemed of value to investigate its method of action. The nature of the reaction during which methemoglobin is formed is readily available for study, and it has been the purpose of this work to determine whether this function of *Streptococcus viridans* is the same as that possessed by the pneumococcus.

[1] Cole, R., *J. Exp. Med.*, 1914, xx, 363.

Methods.

Measured amounts of cultures of *Streptococcus viridans* were added to 1 cc. of a 5 per cent normal saline suspension of washed sheep red blood corpuscles in small test-tubes under many different environmental conditions. These mixtures with suitable controls were incubated in a water bath at 37°C. for varying periods of time as the requirements of the experiments demanded. Methemoglobin formation was considered to have taken place when the red color of oxyhemoglobin was changed to the brown color of methemoglobin, and the results have been recorded throughout as follows: ++ indicates complete transformation of oxyhemoglobin to methemoglobin; + indicates moderate methemoglobin formation; +− indicates slight methemoglobin formation; − indicates no demonstrable change.

Preliminary experiments showed that the most satisfactory culture medium for use was plain ascitic bouillon (peptone 2 per cent, bouillon 4 parts, ascitic fluid 1 part, reaction 0.3+ to phenolphthalein) and this has been used throughout. Such a medium in itself has no effect on sheep red blood corpuscles; *Streptococcus viridans* grows abundantly and rapidly in it; and an acidity sufficient in itself to transform oxyhemoglobin into methemoglobin is never produced, such as occurs when a culture medium containing dextrose is used.

Many different strains of *Streptococcus viridans* have been tested, isolated from various sources, such as acute tonsillitis, bronchopneumonia, malignant endocarditis, alveolar abscesses, etc. No significant difference in the action of different strains has occurred.

EXPERIMENTAL.

To determine whether living streptococci were necessary to cause methemoglobin formation, parallel tests with living cultures, cultures killed by exposure to 58°C. for 30 minutes, and with culture medium from which the streptococci had been removed by centrifugalization were made. The results are shown in Table I.

These experiments have been repeated many times and have constantly shown that methemoglobin is formed only in the presence

TABLE I.

Tube No.	Material used.	Methemoglobin formation, 2 hrs. at 37°C.
1	1 cc. Culture E3 + 1 cc. suspension of sheep corpuscles.	++ in 10 min.
2	1 cc. Culture E3 (heated at 58°C. for 30 min.)+ 1 cc. suspension of sheep corpuscles.	–
3	1 cc. Culture E3 (streptococci removed) + 1 cc. suspension of sheep corpuscles.	–
4	1 cc. sterile culture media + 1 cc. suspension of sheep corpuscles.	–

Culture E3 is an 18 hr. ascitic bouillon culture of *Streptococcus viridans* E3 from a case of malignant endocarditis.

of living streptococci and not by killed organisms or by any substance produced by the growth of the bacteria in the culture medium and separable from them.

To determine the optimum period of growth for the production of methemoglobin and to discover whether this function of *Streptococcus viridans* persisted as long as the organisms were viable, or was associated only with multiplication of the bacteria, the following experiment was done. Measured amounts of culture were added to washed sheep corpuscles at frequent intervals up to 60 hours after inoculation of the culture medium, and results recorded in the usual way as shown in Table II.

The formation of methemoglobin occurred after the culture had been incubated for 6 hours and continued at its height up to 18 hours. After this time the intensity of the reaction gradually diminished and finally disappeared after 60 hours' incubation. Subcultures made at this time showed the culture to be still viable. Numerous similar experiments have yielded like results with some slight variation in the optimum period for the most active formation of methemoglobin. These variations have apparently depended upon the rapidity of growth of a given strain. From these experiments it seems probable that the reaction is associated with multiplication of the streptococci, being most intense during the period of greatest growth and

TABLE II.

Tube No.	Culture T1.	Salt solution.	Sheep corpuscles.	Methemoglobin formation, 1 hr. at 37°C., after incubation of Culture T1 from 3 to 60 hrs. Hours.								
				3	6	9	14	18	23	30	48	60
	cc.	*cc.*	*cc.*									
1	1.0	—	1.0	—	++	++	++	++	++	+	+	—
2	0.9	0.1	1.0	—	++	++	++	++	++	+	+	—
3	0.8	0.2	1.0	—	++	++	++	++	+	+	+−	—
4	0.7	0.3	1.0	—	++	++	++	++	+	+	+−	—
5	0.6	0.4	1.0	—	+	++	++	++	+	+−	+−	—
6	0.5	0.5	1.0	—	+	+	+	+	+−	+−	+−	—
7	0.4	0.6	1.0	—	+−	+	+	+	+−	+−	+−	—
8	0.3	0.7	1.0	—	—	+−	+−	+−	+−	—	—	—
9	0.2	0.8	1.0	—	—	+−	+−	+−	—	—	—	—
10	0.1	0.9	1.0	—	—	—	—	—	—	—	—	—
11	—	1.0	1.0	—	—	—	—	—	—	—	—	—
12	1. cc. of sterile culture media.	—	1.0	—	—	—	—	—	—	—	—	—

gradually disappearing as further multiplication ceases, though the culture still remains viable.

In testing various strains of *Streptococcus viridans* in the preceding experiment it soon became evident that there was considerable variation in the ability of various strains to form methemoglobin. Much larger amounts of culture were required with some strains than with others to cause complete transformation of oxyhemoglobin to methemoglobin when tested against a constant amount of sheep corpuscles. It seemed possible that this variability might bear some relation to the virulence of the strains or to the sources from which they were isolated. Accordingly titration of a considerable number of strains was done under uniform conditions. Diminishing amounts of 12 hour ascitic bouillon cultures were added to 1 cc. of washed sheep corpuscles, and incubated for 1 hour at 37°C. The smallest amount of culture which caused complete transformation of oxyhemoglobin to methemoglobin was taken as a measure of the activity of a given strain and may be spoken of as the methemoglobin titer of that strain. A tabulation of the results obtained, with the source and virulence of the strains for comparison, is shown in Table III.

TABLE III.

Culture.	Methemoglobin titer.	Source.	Virulence for rabbits.*	
			Amount of culture.	Effect.
	cc.		*cc.*	
AA3b3....	1.0	Alveolar abscess.		
T12d.....	1.0	Acute tonsillitis.	12	No effect.
			40	Septicemia; death in 18 hrs.
SF1.....	0.9	Throat, scarlet fever.	5	No effect.
R1.....	0.8	" rheumatic fever.	40	Arthritis.
T5.....	0.7	Acute tonsillitis.	10	Septicemia; death in 20 hrs.
T14.....	0.7	" "	4	No effect.
			12	Septicemia; death in 18 hrs.
AA3a.....	0.6	Alveolar abscess.		
AA3b1....	0.6	" "		
T1.....	0.6	Acute tonsillitis.	5	Acute pericarditis, acute myocarditis, arthritis.
AA3b4....	0.5	Alveolar abscess.		
D1a.	0.5	Throat, diphtheria.		
AA1k.....	0.5	Alveolar abscess.	5	Aortic endocarditis, myocarditis, arthritis; death in 18 days.
P8.....	0.4	Pneumonia.	2	No effect.
P10.....	0.4	"	5	Arthritis.
AA1.....	0.4	Alveolar abscess.	2	Septicemia; death in 24 hrs.
AA1j.....	0.4	" "		
T3.....	0.3	Acute tonsillitis.	60	Mitral endocarditis, appendicitis, arthritis; death in 9 days.
T4.....	0.3	" "	15	Septicemia; death in 24 hrs.
T14b	0.3	" "		
E12b.....	0.3	Blood, endocarditis.		
T11.....	0.2	Acute tonsillitis.	5	Acute pericarditis, acute arthritis; death in 24 hrs.
T11a.....	0.2	Abscess, pyemia.		
D1c.....	0.2	Throat, diphtheria.		
P3.....	0.2	Pneumonia.	4	Septicemia; death in 18 hrs.
AA2.....	0.2	Alveolar abscess.	8	No effect.
E3.....	0.2	Blood, endocarditis.	50	Arthritis.
E12v2.....	0.2	Mitral valve, endocarditis.		
T12b.....	0.1	Acute tonsillitis.		
T12c.....	0.1	" "		

*Rabbit inoculations were given intravenously. Measured amounts of 24 hr. dextrose bouillon cultures were centrifuged and the bacterial sediment was suspended in salt solution before inoculation.

Study of the results shows no relation between the source and virulence of the different strains of *Streptococcus viridans* and their methemoglobin titers. Similar results have been found for hemolytic streptococci by Lyall.[2] On the other hand, the titer of our strains seemed to vary largely with the abundance and rapidity of growth of a given strain, thus lending further support to the theory that the formation of methemoglobin is a function associated with multiplication of the streptococci.

If the formation of methemoglobin is the result of the metabolic activities occurring during the growth and multiplication of the streptococci, the reaction should take place only under those conditions which are suitable for multiplication; that is, in the presence of nutrient material. The following experiment has shown this to be true. An 18 hour ascitic broth culture of streptococci was centrifuged, the supernatant fluid removed, and the bacterial sediment thoroughly washed and finally suspended in salt solution, the suspension being prepared so that the streptococci were in twice as great concentration as in the original culture. This suspension was tested against sheep corpuscles alone and with the addition of ascitic bouillon, plain bouillon, ascitic fluid, meat infusion, peptone, and dextrose as shown in Table IV.

It was evident from these experiments that some nutrient substance is necessary for the formation of methemoglobin. Repetition of these experiments with increasing dilutions of the various substances showed that relatively large amounts of ascitic fluid, meat infusion, and peptone were required for the reaction, but that methemoglobin was formed rapidly in the presence of dextrose in great dilution, even in as small amounts as 0.5 cc. of a 1:1,000 dilution of a 5 per cent dextrose solution. Since the presence of such minute traces of dextrose is sufficient to bring about the reaction, it seems possible that traces of sugar may have been present in the peptone solution and have been responsible for the reaction in that case. Certainly this may have been so in the experiments with ascitic fluid and meat infusion.

Various kinds of sugars and other substances were next tested to determine whether streptococci would produce methemoglobin in their

[2] Lyall, H. W., *J. Med. Research*, 1914, xxx, 515.

TABLE IV.

Tube No.	Material used.	Methemoglobin formation, 2 hrs. at 37°C.
1	0.5 cc. bacterial suspension T1 + 1 cc. sheep corpuscles + 0.5 cc. salt solution.	−
2	0.5 " " " T1 + 1 " " " + 0.5 " ascitic bouillon.	++
3	0.5 " " " T1 + 1 " " " + 0.5 " plain "	++
4	0.5 " " " T1 + 1 " " " + 0.5 " ascitic fluid.	++
5	0.5 " " " T1 + 1 " " " + 0.5 " meat infusion.	++
6	0.5 " " " T1 + 1 " " " + 0.5 " peptone solution.	+
7	0.5 " " " T1 + 1 " " " + 0.5 " dextrose "	++
8	0.5 " salt solution + 1 " " " + 0.5 " ascitic bouillon.	−
9	0.5 " " " + 1 " " " + 0.5 " plain "	−
10	0.5 " " " + 1 " " " + 0.5 " ascitic fluid.	−
11	0.5 " " " + 1 " " " + 0.5 " meat infusion.	−
12	0.5 " " " + 1 " " " + 0.5 " peptone solution.	−
13	0.5 " " " + 1 " " " + 0.5 " dextrose "	−
14	0.5 " Culture T1 + 1 " " " + 0.5 " salt "	++

The peptone solution consisted of 5 per cent solution of Witte's peptone in distilled water, autoclaved for 15 minutes at 15 pounds' pressure.

The dextrose solution consisted of 5 per cent solution of chemically pure dextrose in distilled water, autoclaved for 15 minutes at 15 pounds' pressure.

presence as well as in the presence of dextrose. The results are given in Table V. Control tubes without the addition of streptococci showed that none of the test substances cause methemoglobin formation.

Further experiments with increasing dilutions of the various test substances to determine the minimum amount of each substance in the presence of which the reaction would occur showed considerable variation not only in the amount required but also in the rapidity of the change of oxyhemoglobin to methemoglobin. No definite relationship could be determined between these variable results and the molecular configuration of the substances used, although the reaction was usually more rapid and occurred in the presence of higher dilutions of dextrose, lactose, saccharose, maltose, and levulose than of the other substances. Furthermore, there seemed to be no relationship between the carbohydrate cleavage power of a strain

TABLE V.

Test substance.	Bacterial suspension.	Sheep corpuscles.	Methemoglobin formation.		
			T3.	P 10.	E3.
	cc.	*cc.*			
0.5 cc. 3 per cent inulin solution.....	0.5	1.0	+	++	++
0.5 " 5 " " lactose "	0.5	1.0	++	++	+−
0.5 " 5 " " mannite "	0.5	1.0	+	++	−
0.5 " 5 " " raffinose "	0.5	1.0	++	++	++
0.5 " 5 " " saccharose "	0.5	1.0	+	++	++
0.5 " 5 " " salicin "	0.5	1.0	+−	+	+−
0.5 " 5 " " galactose "	0.5	1.0	+	++	−
0.5 " 5 " " maltose "	0.5	1.0	++	++	++
0.5 " 5 " " dextrin "	0.5	1.0	++	++	++
0.5 " 5 " " levulose "	0.5	1.0	++	++	++
0.5 " 5 " " starch "	0.5	1.0	++	+	+
0.5 " 5 " " glycerine "	0.5	1.0	−	++	+
0.5 " 5 " " urea "	0.5	1.0	−	−	−
0.5 " 10 " " methyl alcohol.....	0.5	1.0	−	−	−
0.5 " 10 " " ethyl "	0.5	1.0	+	++	+

All the tubes were incubated at 37° C. for 2 hrs. T3, P10, and E3 are different strains of *Streptococcus viridans.*

of streptococcus and its ability to form methemoglobin in the presence of various carbohydrates. Of six strains which formed methemoglobin in the presence of inulin, three fermented the sugar, three did not; and of five strains causing methemoglobin formation in the presence of mannite, only one fermented that substance.

Since methemoglobin may be formed by streptococci in the presence of sugars which the organisms do not ferment, it seems improbable that the reaction depends upon intermediate or end-products formed by the action of the streptococci on the nutrient substance present. Certainly the reaction cannot be due to the formation of carbon dioxide, for carbon dioxide changes oxyhemoglobin to a cherry-red color and such a change is never caused by *Streptococcus viridans.* Neither can the reaction be due to the production of acid, for if experiments such as the above are carried out with the addition of a mixture of primary and secondary potassium phosphates so that neutrality of the mixture is maintained, not only does the formation of methemoglobin still take place, but in many instances it is acceler-

ated. Cole[1] has shown that many substances which are supposed to represent the intermediate and end-products of sugar metabolism are incapable of producing methemoglobin except in much more concentrated solutions than could possibly occur in the above experiments. It is conceivable that some of these substances might be sufficiently active in a nascent state to bring about the reaction, but this is hardly probable.

To determine the effect of the absence of oxygen on the reaction the following experiment was done.

A stream of hydrogen was passed through a suspension of red blood corpuscles for 10 minutes and the suspension then covered with paraffin oil. An ascitic bouillon culture of streptococci was treated in the same manner. A measured amount of the culture was then drawn into a pipette which had been passed through the oil to the bottom of the culture tube and was immediately added to the suspension of red blood cells, the pipette being passed through the oil to the bottom of the tube containing the corpuscle suspension. No air was allowed to enter the mixture. Mixtures so prepared showed no methemoglobin formation at the end of 2 hours' incubation, while in the untreated control tubes complete transformation of oxyhemoglobin to methemoglobin took place in 10 minutes. A stream of oxygen was now passed through the mixture previously treated with hydrogen, with the result that methemoglobin formation rapidly occurred. Two tubes were then prepared each containing a like mixture of the corpuscle suspension and an ascitic bouillon culture of streptococci. Through one a stream of oxygen was passed, through the other a stream of air. The tubes were incubated together. Under these conditions methemoglobin formation in the tube subjected to an excess of oxygen was considerably delayed. These experiments show that methemoglobin formation by *Streptococcus viridans* does not occur in the absence of oxygen and that it is retarded in the presence of an excess of oxygen.

It seemed possible that some further knowledge of the nature of the reaction during which methemoglobin is formed might be gained by studying the inhibitory action of various substances. Experiments were done in which diminishing amounts of toluol, formaldehyde, sodium salicylate, salicylic acid, potassium carbonate, potassium iodide, alcohol, and chloroform, and various sera were added to the culture-sheep-corpuscle mixtures. The results in general were not of sufficient significance to make it profitable to present them in detail. In brief it may be said that toluol (1:20), formaldehyde (1:2,000), 5 per cent sodium salicylate solution, and 20 per cent saturated salicylic acid solution had no appreciable effect on the reaction. Chloroform in the proportion of 1:20, ethyl alcohol in

the proportion of 1:10, 100 per cent potassium iodide solution in the proportion of 1:5, and 25 per cent potassium carbonate solution in the proportion of 1:5 caused complete inhibition of the reaction. Inhibition of methemoglobin formation in the presence of these substances may be attributed to a reduction of the metabolic activities of the organisms to a minimum.

The results with various sera were interesting but threw little light on the mechanism of the reaction in question. Normal human sera as a rule caused definite retardation and partial inhibition of the reaction (Table VI).

TABLE VI.

	cc.	cc.	cc.	cc.	cc.	cc.	cc.	cc.	cc.	cc.
Culture P3 . . .	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	–	0.1
Human serum.	0.5	0.4	0.3	0.2	0.1	0.5 (1:10)	0.3 (1:10)	0.1 (1:10)	0.5	–
Salt solution . .	0.4	0.5	0.6	0.7	0.8	0.4	0.6	0.8	0.5	0.9
Sheep corpuscles.	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Methemoglobin formation at 37°C.										
30 min.	–	–	+–	+–	+–	+	+	+	–	+
1 hr.	+–	+–	+	+	+	++	++	++	–	++

In other instances the result was much less marked. Lyall[2] has called attention to the inhibiting effect of salvarsanized serum on the action of hemolytic streptococci. In our experiments salvarsanized serum was not more efficacious than normal human serum in its inhibitory action on methemoglobin formation. Normal sheep serum and guinea pig serum had a moderate inhibiting action, while normal rabbit serum had little effect.

Two experiments with sera from patients with streptococcus septicemia are of interest in this connection. A culture of *Streptococcus viridans* E3 obtained from the blood of a patient with malignant endocarditis was tested in the usual manner against sheep corpuscles with the addition of diminishing amounts of the patient's serum, a parallel series of tubes with normal human serum being run at the same time as a control (Table VII). Complete inhibition of methemoglobin formation took place in those tubes to which the patient's serum had been added.

TABLE VII.

Culture E3.	Salt solution.	Sheep corpuscles.	Serum.	Methemoglobin formation at 37°C.			
				Patient's serum.		Normal human serum.	
				30 min.	1 hr.	30 min.	1 hr.
cc.	*cc.*	*cc.*	*cc.*				
0.3	0.2	1.0	0.5	−	−	+−	++
0.3	0.3	1.0	0.4	−	−	+−	++
0.3	0.4	1.0	0.3	−	−	+	++
0.3	0.5	1.0	0.2	−	−	+	++
0.3	0.6	1.0	0.1	−	−	++	++
0.3	0.2	1.0	0.5 (1:10)	−	−	++	++
0.3	0.4	1.0	0.3 (1:10)	−	−	++	++
0.3	0.6	1.0	0.1 (1:10)	−	−	++	++
0.3	0.7	1.0	−	++	++	++	++
−	0.5	1.0	0.5	−	−	−	−

The second experiment in which similar results were obtained was with a hemolytic streptococcus isolated from the blood in a case of septicemia following tonsillectomy. Nearly complete inhibition of hemolysis occurred in the series of tubes to which the patient's serum had been added. This inhibition of the metabolic activities of the streptococci by serum may be concerned with the phenomena of antiblastic immunity as described by Dochez and Avery.[3]

SUMMARY AND CONCLUSIONS.

Cultures of *Streptococcus viridans* when brought into contact with red blood corpuscles have the power of transforming oxyhemoglobin into methemoglobin. The reaction occurs only in the presence of living streptococci when they are able to carry on their metabolic activities. The intensity of the reaction runs roughly parallel with the period of growth and multiplication of the bacteria and gradually diminishes and disappears as growth ceases. There is no apparent relation between the activity of a given strain of *Streptococcus viridans* in producing methemoglobin and its source or virulence. If the streptococci are suspended in salt solution they are unable to change

[3] Dochez, A. R., and Avery, O. T., *J. Exp. Med.*, 1916, xxiii, 61.

oxyhemoglobin into methemoglobin unless some nutrient substance is present. Of the various nutrient substances tested dextrose is the most efficient in enabling the organisms to bring about the reaction. The reaction does not occur in the absence of oxygen, and is retarded by an excess of oxygen. Substances which tend to reduce the metabolic activities of the bacteria to a minimum exert an inhibitory action on methemoglobin formation. While not definitely proving it to be so, the results obtained in the above experiments strongly support the supposition that the reaction is not due to injurious substances produced by the bacteria or to products arising from the decomposition of the nutrient material present, but rather to the metabolic activities of the bacteria themselves when they are surrounded by environmental conditions which render growth and multiplication possible.

The exact chemical nature of the change of oxyhemoglobin to methemoglobin is not known, but it is probably an oxidation process or a combination of reduction and oxidation processes, as pointed out by Heubner.[4] As Cole[1] has shown, the action of aminophenol is of great interest in this connection, in that it acts like a catalytic agent in being able to transform much more hemoglobin into methemoglobin than would be possible if the reaction were a simple molecular one.

The metabolic activities of bacteria are largely in the nature of oxidation and reduction processes. The transformation of oxyhemoglobin into methemoglobin by streptococci of the *viridans* type, therefore, may be analogous to the action of such substances as aminophenol, and the reaction may be due to the active oxidation and reduction processes occurring in the neighborhood of the bacterial cells. The failure of the reaction to occur in the absence of oxygen and its retardation in the presence of an excess of oxygen, both with streptococci and with pneumococci (Cole[1]) would seem to support this theory. Such results, however, may be due to the abnormal conditions surrounding the bacteria with consequent inhibition of their metabolic activities. Cole[1] concluded as the result of his study of methemoglobin formation by pneumococci that since

[4] Heubner, W., *Arch. exp. Path. u. Pharm.*, 1913, lxxii, 239.

bacteria may injure red blood cells apparently by disturbances in oxidation in the immediate neighborhood of the organisms rather than by the production of a definite toxin, it is possible that bacteria may injure other tissue cells in a like manner and that the pathological effects produced by these bacteria may be explained on this basis. The experimental results recorded above have shown that the formation of methemoglobin by *Streptococcus viridans* in no way differs from its formation by pneumococci, and they lend support to the theory that bacteria may be injurious to tissues because of the disturbances in oxidation brought about by the metabolic activities of the organisms, especially those associated with growth and multiplication. It is believed that this theory may be particularly applicable to the pathological effects caused by *Streptococcus viridans* because the lesions produced by it, whether single or multiple, both in man and in experimental animals, are prone to be localized and associated with the actual presence of the streptococci in the lesions.

A FURTHER STUDY OF THE GASTRIC ULCERS FOLLOWING ADRENALECTOMY.

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PLATES 36 AND 37.

(Received for publication, June 1, 1916.)

In a previous study of adrenalectomized animals,[1] the frequent occurrence of acute ulceration of the gastric mucosa and the occasional occurrence of duodenal ulcer were noted. While these ulcers were not found in adrenalectomized animals subjected to continuous etherization, and were infrequent in animals subjected to the removal of only one gland, they occurred in about 90 per cent of those dying under the characteristic symptoms of adrenal insufficiency after the removal of both glands. The ulcers developed during the moribund period, were apparently peptic, forming at the site of the local hemorrhages in the gastric mucosa, and were true acute ulcers, usually penetrating to the muscularis mucosa, with a total loss of epithelium. While they occurred in the absence of pancreatic secretion and bile, they appeared to develop only in an acid medium.

In order to determine whether the acid medium was the important factor in the production of the acute ulcers, or whether their cause resided in other factors, such as the special nerve or vascular mechanism of the stomach, the following experiments were performed.

A loop of the first part of the jejunum, varying in length from 6 to 12 cm., was functionally resected under anesthesia and implanted in the posterior wall of the stomach in the region of the antrum pylori. The continuity of the intestine was maintained by an intestinal anastomosis.

[1] Mann, F. C., A Study of the Gastric Ulcers Following Removal of the Adrenals, *J. Exp. Med.*, 1916, xxiii, 203.

Dogs operated on in this manner quickly recovered from the operation and maintained excellent health for many months. Animals killed at various periods after operation demonstrated that the transplanted mucosa underwent definite changes, but ulcers did not develop except in one instance in which an ulcer was found around a retained silk suture.

In four of these animals, after a considerable length of time had elapsed, the right adrenal was removed, and a few months later the left gland was extirpated. All developed the typical symptoms of adrenal insufficiency and died at various times after the removal of the last gland.

In all these animals definite lesions of the gastric mucosa were found at autopsy. In three, there were ulcerations, while in one the mucosa was injected only and showed areas of erosion. In three the jejunal transplant appeared exactly similar to the control. In one there appeared to be a slight loss of jejunal mucosa in pin-point areas, which microscopically proved to be small ulcers.

It was hoped that these experiments would prove clearly whether or not free acidity was the primary cause in the formation of these ulcers. If acidity is the primary cause, the jejunal mucosa, to which an acid medium is foreign, should show the most marked changes. If the primary cause lies in the intrinsic mechanism of the gastric mucosa, the latter alone should be involved.

The results of the experiments show that both factors are of importance. In the three experiments in which the gastric mucosa alone was involved, the transplant having been left intact, either the ulcerations and erosions were due to a primary impairment of the gastric mucosa to which the jejunal mucosa was not subjected, or the acidity developed within the gland tubules and produced its destructive action there first. If the latter is true, the jejunal mucosa might become involved afterwards. The experiment in which the transplanted mucosa was involved might be interpreted in this way. The evidence tends to show that the acidity is but a secondary, although necessary factor and that the primary cause lies in the intrinsic mechanism of the gastric mucosa. However, the fact that changes in the jejunal mucosa occurred in one experiment shows the importance of acidity.

Protocols.

Dog 1.—Under ether anesthesia a jejunal transplant was made on June 29, 1915. The animal quickly recovered from the effects of the operation and its health was excellent. Killed Oct. 19.

Autopsy.—The anastomosis and transplant were in good condition. The transplant showed thickening of the muscularis mucosa as compared with the control part of the jejunum. The size of the transplant was 7.5 by 3 cm. The mucosa of the stomach and of the transplant was otherwise negative (Fig. 1).

Dog 2.—Under ether anesthesia a jejunal transplant was made on Oct. 14, 1915. On Feb. 8, 1916, the right adrenal was removed. The condition of the animal was always excellent. On Apr. 11, the left adrenal was removed. The animal died from adrenal insufficiency on Apr. 21.

Autopsy.—The usual findings in animals dying from adrenalectomy were noted. The stomach was contracted and contained about 10 cc. of bile-stained, acid fluid. The gastric mucosa was injected throughout. In the pyloric region were four ulcers which measured 4 mm. in diameter, and probably extended to the muscularis mucosa. The transplant was 4 by 2.2 cm., and did not differ in any respect from the controls (Fig. 2).

Dog 3.—Under ether anesthesia a jejunal transplant was made on Oct. 12, 1915. On Feb. 8, 1916, the right adrenal was removed. The condition of the animal was always excellent. On Apr. 16, the left adrenal was removed. The animal died from adrenal insufficiency on Apr. 21.

Autopsy.—The usual findings of adrenalectomized animals were noted. The stomach contained 10 cc. of bile-stained, acid fluid. The gastric mucosa was bile-stained and injected throughout. In the pyloric mucosa was one small ulcer. At the edge of the transplant in the gastric mucosa a large ulcer was found. The latter, however, may have been partially due to a retained suture. The transplant was 8 by 2.5 cm., and appeared in every respect similar to the controls (Fig. 3).

Dog 4.—Under ether anesthesia a jejunal transplant was made on Oct. 29, 1915. On Feb. 8, 1916, the right adrenal was removed. The condition of the animal was always excellent. On Apr. 16, the left adrenal was removed. The animal died on Apr. 21 from adrenal insufficiency.

Autopsy.—The findings characteristic of an adrenalectomized animal were noted. The stomach contained a small amount of bile-stained, acid fluid. There were no frank gastric ulcers, but the mucosa was markedly injected and there were many areas of erosion. The transplant was 3 by 1.5 cm., and appeared similar to the controls.

Dog 5.—Under ether anesthesia a jejunal transplant was made on Oct. 13, 1915. The animal continued in excellent health. On Feb. 8, 1916, the right adrenal was removed. The animal remained in perfect condition. The left adrenal was removed on Apr. 24. The animal died from adrenal insufficiency on May 2.

Autopsy.—The usual findings in animals dying after adrenalectomy were noted. The stomach contained about 50 cc. of bile-stained, acid fluid. The fundal mucosa was markedly injected and contained many ulcers varying from 0.2 to 0.8 cm. in diameter. The pyloric mucosa showed a few ulcers. In the transplant were a few pitted areas which microscopically proved to be small ulcers. The size of the transplant was 3.5 by 3 cm. (Fig. 4).

SUMMARY.

Acute gastric ulcers are found in a large percentage of animals dying from acute adrenal insufficiency. In dogs in which a portion of the jejunum had been transplanted some time previous to the removal of the adrenals, the gastric mucosa showed more marked changes than the transplanted jejunal mucosa. This tends to show that the gastric juice as the cause of the ulcer is but a secondary although necessary factor.

EXPLANATION OF PLATES.

PLATE 36.

FIG. 1. Gastric and jejunal mucosa of Dog 1. This shows the characteristic appearance of the transplant in normal dogs.

FIG. 2. Gastric mucosa and transplant of Dog 2. Note the ulcers in the pyloric mucosa and the normal appearance of the transplanted jejunal mucosa.

PLATE 37.

FIG. 3. Gastric mucosa and transplant of Dog 3. The large ulceration is probably due to a retained silk suture. Note the injection of the gastric mucosa as compared with the jejunal transplant.

FIG. 4. Gastric mucosa and transplant of Dog 5. Note the marked injection, erosion, and ulceration of the gastric mucosa and the pin-point erosions of the transplanted jejunal mucosa.

FIG. 1.

FIG. 2.

(Mann: Gastric Ulcers Following Adrenalectomy.)

FIG. 3.

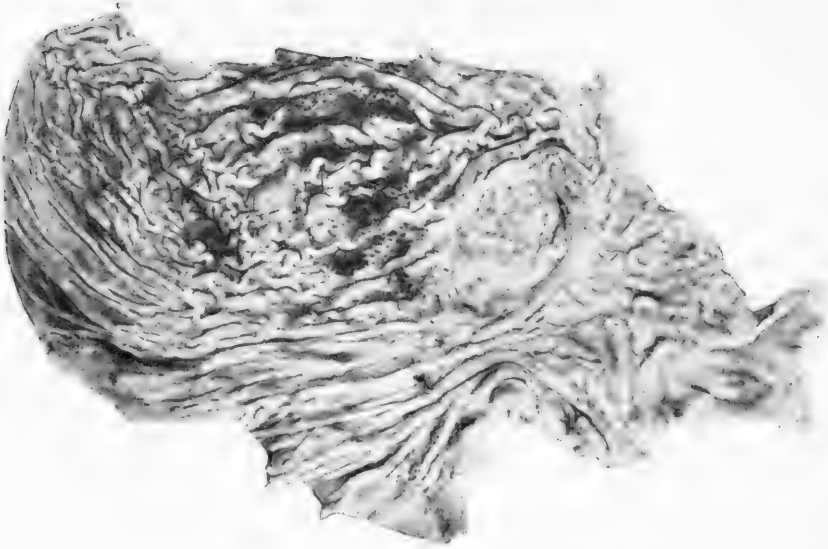

FIG. 4.

(Mann: Gastric Ulcers Following Adrenalectomy.)

AGGLUTINATION OF TREPONEMA PALLIDUM IN HUMAN SYPHILIS.

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In 1913 one of us (1) first described agglutination of a pure culture of *Treponema pallidum* by the sera of rabbits injected with a living and heat-killed culture.[1] Normal rabbit sera were found not to agglutinate this culture in dilutions as low as 1:20 (lower dilutions were not used), while the sera of immunized animals were found capable of agglutinating the culture in dilutions as high as 1:1,280. The agglutinating power of the sera of nineteen individuals in various stages of syphilis and of ten non-syphilitic individuals was studied in dilutions ranging from 1:20 to 1:640, but with negative results, due in large part, as we now believe, to the fact that lower dilutions of serum were not employed.

Previous to this, attempts had been made by Hoffmann and von Prowazek (2), Herxheimer and Löser (3), Hoffmann (4), Brönnum and Ellermann (5), Babes and Panea (6), Metchnikoff and Roux (7), Landsteiner and Mucha (8, 9), Zabolotny and Maslakowetz (10), and Uhlenhuth and Mulzer (11) to determine the presence of agglutinins for *Treponema pallidum* in the sera from human and experimental syphilis according to the grouping of the spirochetes in the tissues and with extracts and emulsions of tissues rich in the microparasites. While the presence of an agglutinin was suspected, technical difficulties, owing to the emulsion employed in the absence of pure cultures, did not permit the deduction of any positive conclusions.

In 1913, Nakano (12) found agglutinins for a culture of *Treponema pallidum* in the sera of rabbits injected intravenously with a heat-killed culture, that yielded well marked agglutination in dilutions from 1:10 to 1:70. Kissmeyer (13) working with a pure culture reported that normal human serum may agglutinate *Treponema pallidum* in dilutions as high as 1:50; he also tested the sera of 59 individuals in the various stages of syphilis and reported positive results in dilutions of 1:100 and higher in about 40 to 60 per cent of cases in the primary, secondary, and tertiary stages, including congenital syphilis, while the sera of immunized rabbits were reported as yielding well marked agglutination in dilutions

[1] The culture was obtained from Dr. Hideyo Noguchi of The Rockefeller Institute for Medical Research.

ranging from 1: 2,000 to 1: 200,000. Zinsser and Hopkins (14) have found that normal rabbit serum may agglutinate *Treponema pallidum* in dilutions of 1: 10 and lower, and the sera of immunized animals as high as 1: 2,000. They also stated that "normal human sera will agglutinate similar *pallidum* emulsions, as will the sera of certain syphilitic patients with positive Wassermann reactions," but gave no details of this portion of their work. In a second paper Zinsser, Hopkins, and McBurney (15) present important evidence indicating that the immune agglutinins in the sera of immunized rabbits and sheep failed to cause agglutination of virulent *pallida* from human lesions.

In this communication we desire to give the results of a study of agglutination of a pure culture of *Treponema pallidum* by normal human sera, the sera of persons in the various stages of syphilis, and the sera of non-syphilitic persons suffering with various other diseases.

Method.

Sera.—The sera of 12 persons known not to be syphilitic and with negative Wassermann reactions were studied; also the sera of 5 persons late in the primary, and 12 in the secondary stages of syphilis; 22 from patients with paresis; 12 from patients with positive Wassermann or luetin reactions and regarded as being tertiary or latent syphilitics; and 8 from persons with negative Wassermann reactions and denying syphilitic infection, but suffering with various other infections at the time when these tests were made.

Culture.—All the tests here reported were conducted with Zinsser's Strain A culture of *Treponema pallidum*. This culture was received growing under paraffin oil in ascitic broth over an egg medium and has been successfully grown in large tubes of egg medium covered with ascitic broth and paraffin oil. This method has proven very satisfactory, and luxuriant growths of active treponemata are secured uniformly within a 4 weeks' period of incubation.

After removal of the paraffin oil the fluid cultures in each tube were removed to sterile flasks with particular care not to break up the solid media and to remove the whitish sediment of treponemata which collects in the angle between the slant of egg media and the wall of the test-tube. After shaking the emulsions with sterile glass beads and centrifuging briefly, they were ready for use. Living cultures were employed throughout, and the emulsions were of such

density that each microscopic field examined by dark-field illumination showed from 12 to 30 treponemata.

Technique.—The macroscopic test was employed in exactly the same manner as in the previous study by Kolmer, except that lower dilutions were used; namely, 1:2, 1:5, 1:10, 1:20, 1:40, and 1:80.

1 cc. of treponema emulsion was used in each test, the serum being diluted in 1 cc. of sterile saline solution; the total volume, therefore, being 2 cc. with this amount of culture, a sufficient number of spirochetes were present to yield unmistakable agglutination in positive reactions. The first dilution (1:2) was prepared by adding 1 cc. of emulsion to 1 cc. of serum; further dilutions of serum in 1 cc., as 1:2.5, 1:5, 1:10, 1:20, and 1:40, were prepared, which after the addition of 1 cc. of emulsion were doubled in volume.

All the sera were unheated in dilutions varying from 1:2 to 1:80. After the preparations were set up the tubes were incubated for 2 hours at 37°C. and then placed in the refrigerator over night, final readings being made the next morning.

In every instance culture controls in normal saline solution were included; there were no evidences of spontaneous agglutination. As pointed out in the former communication, two or three spirochetes may be found in a loose clump, but in no instance were the large, clearly defined clump of spirochetes found, as in positive agglutinations.

In all tests a microscopic examination with the dark-field illuminator was made. Occasionally a light precipitate due to constituents in the culture medium may collect in the bottom of the small test-tubes, which macroscopically may be mistaken for agglutinated spirochetes; furthermore, the emulsions were not of sufficient density when diluted with an equal quantity of the serum dilution to render reliable a macroscopic reading of the reactions. For these reasons, we have examined each tube microscopically, removing the sediment or supernatant fluid or both with a fine capillary pipette and examining a drop by dark-field illumination.

In every instance the controls and negative reactions showed the presence of a large number of spirochetes, a few still motile, and all free from clumping. In a few instances two or three spirochetes were found in clumps, but there was no sedimentation and this was not

considered as an evidence of agglutination. In all positive reactions a drop of the sediment examined by dark-field illumination showed the presence of large clumps of spirochetes which were in most instances non-motile, although a few motile organisms were to be found at the edge of a clump, or entirely free. In the higher dilutions where agglutination was incomplete, free active spirochetes were found in the supernatant fluid and clumps in the sediment.

Wassermann Reactions.—All sera were submitted to the Wassermann reaction with three lipoidal antigens; namely, an alcoholic extract of human heart reenforced with cholesterol (C.H.), an alcoholic extract of syphilitic liver (S.), and an extract of acetone-insoluble lipoids from human heart muscle (A.).

With a portion of the sera an antigen of treponema in the form of luetin was employed; this antigen was titrated before each experiment and used in an amount equivalent to one-third of the anti-complementary dose.

All reactions were read immediately after the secondary period of incubation.

RESULTS.

Normal Human Sera.—The results observed with twelve sera are shown in Table I.

Half of these sera showed no evidence of agglutination in the lowest dilution; namely, equal parts of undiluted serum and emulsion. As pointed out above, however, we have not designated as agglutination the loose clumps of two or three spirochetes which are occasionally encountered in tests with normal sera and in the controls.

In 50 per cent of these sera there were some evidences of agglutination in the lowest dilution, but none in dilutions of 1: 5 and higher.

In our opinion agglutination of culture *Treponema pallidum* by human sera must occur in dilutions of 1: 5 or higher before any significance may be attached to the result from the standpoint of syphilis.

Sera of Non-Syphilitic Individuals Suffering with Other Infections.—The results observed with eight of these sera are shown in Table II and are similar to those observed with the sera of normal persons shown in Table I. Several of these patients had moderate fever at the time the tests were made, but this factor and the presence of

TABLE I.

Agglutination of Treponema pallidum by the Sera of Normal Individuals.

Case No.	Wassermann reaction.			Agglutination.					
	C. H.*	S.†	A.‡	1:2	1:5	1:10	1:20	1:40	1:80
1	—§	—	—	—	—	—	—	—	—
2	—	—	—	±	—	—	—	—	—
3	—	—	—	—	—	—	—	—	—
4	—	—	—	+	—	—	—	—	—
5	—	—	—	±	—	—	—	—	—
6	—	—	—	—	—	—	—	—	—
7	—	—	—	—	—	—	—	—	—
8	—	—	—	—	—	—	—	—	—
9	—	—	—	+	—	—	—	—	—
10	—	—	—	±	—	—	—	—	—
11	—	—	—	—	—	—	—	—	—
12	—	—	—	±	—	—	—	—	—

* Cholesterolized alcoholic extract of human heart.

† Alcoholic extract of syphilitic liver.

‡ Acetone-insoluble lipoids of human heart.

§ — indicates absence of agglutination; resembles the salt solution controls. ± indicates slight but unmistakable agglutination characterized by numerous clumps of spirochetes; free spirochetes also occur in the supernatant fluid. + indicates well marked agglutination.

TABLE II.

Agglutination of Treponema pallidum by the Sera of Non-Syphilitic Individuals.

Case No.	Diagnosis.	Wassermann reaction.			Agglutination.					
		C. H.	S.	A.	1:2	1:5	1:10	1:20	1:40	1:80
1	Tuberculosis of penis	—	—	—	+	—	—	—	—	—
2	" " skin	—	—	—	—	—	—	—	—	—
3	" " lungs	—	—	—	—	—	—	—	—	—
4	Typhoid fever	—	—	—	±	—	—	—	—	—
5	Lobar pneumonia	—	—	—	±	—	—	—	—	—
6	Pyosalpingitis	—	—	—	—	—	—	—	—	—
7	Pregnancy	—	—	—	—	—	—	—	—	—
8	"	—	—	—	+	—	—	—	—	—

other antibodies in the sera, notably typhoid agglutinin, had no effect upon the spirochetes.

Primary Syphilis.—The results observed with five sera are shown in Table III.

TABLE III.

Agglutination of Treponema pallidum by the Sera of Patients in the Primary Stage of Syphilis.

Case No.	Treatment.	Wassermann reaction.			Agglutination.					
		C. H.	S.	A.	1:2	1:5	1:10	1:20	1:40	1:80
1	1 dose arsenobenzol	++++*	++	++	±	−	−	−	−	−
2	2 doses "	++++	++	+++	+	±	−	−	−	−
3	1 dose "	++	−	−	+	±	−	−	−	−
4	None.....	+	−	−	−	−	−	−	−	−
5	"	++	−	+	±	−	−	−	−	−

* ++++ indicates complete inhibition of hemolysis (strongly positive); +++, 75 per cent inhibition of hemolysis (moderately positive); ++, 50 per cent inhibition of hemolysis (weakly positive); +, 25 per cent inhibition of hemolysis (very weakly positive); ±, 10 per cent inhibition of hemolysis (doubtfully positive).

The first three patients were tested late in the primary stage and after treatment with arsenobenzol had been instituted; Cases 4 and 5 were tested about 2 weeks after the appearance of the chancre and before the treatment was begun.

In all cases the Wassermann reaction was positive; in Nos. 3 and 4 the reaction was positive with the cholesterolized extract only.

While we cannot draw conclusions from the examination of so few cases in this stage of syphilis, it would appear that traces of agglutinin for *Treponema pallidum* may be found in the serum in certain cases late in the primary stage. This opinion is based upon the higher percentage of partial agglutinations in the 1:2 dilution as compared with the sera of normal persons, and upon the occurrence of partial agglutination in the 1:5 dilution in two of the five sera.

Secondary Syphilis.—The results observed with the sera of twelve persons are shown in Table IV.

TABLE IV.

Agglutination of Treponema pallidum by the Sera of Patients in the Secondary Stage of Syphilis.

Case No.	Treatment.	Wassermann reaction.			Agglutination.					
		C. H.	S.	A.	1:2	1:5	1:10	1:20	1:40	1:80
1	1 dose arsenobenzol	++++	++	++	+	+	±	−	−	−
2	1 " "	+	−	−	+	−	−	−	−	−
3	None	++++	++++	++++	+	±	−	−	−	−
4	2 doses arsenobenzol	++++	++++	++++	±	−	−	−	−	−
5	2 " "	++++	++	++++	−	−	−	−	−	−
6	2 " "	++++	+++	++++	+	+	+	+	−	−
7	Mixed (2 wks.)	++++	++++	++++	+	±	−	−	−	−
8	None	++++	++++	++++	+	−	−	−	−	−
9	6 doses arsenobenzol	−	−	−	−	−	−	−	−	−
10	4 " "	+++	++	++	+	±	−	−	−	−
11	None	++++	++++	++++	+	+	−	−	−	−
12	"	++++	++++	++++	+	+	±	−	−	−

All but four of these patients were tested after treatment had been instituted; all but one (Case 9) gave positive Wassermann reactions.

The sera of seven patients showed the presence of agglutinin in dilutions of 1:5 and higher; in no instance, however, was agglutination found in dilutions higher than 1:20.

These results appear to show quite definitely that agglutinins for *Treponema pallidum* are present in the sera of a proportion of cases of syphilis in the secondary stage.

Tertiary Syphilis.—The results observed with the sera of thirty-seven persons regarded as being in the tertiary or late latent stages of syphilis are shown in Tables V and VI.

In Table V are shown the results of agglutination tests with the sera of twenty-one persons regarded clinically as being well developed cases of paresis and of one person in whom the clinical diagnosis was doubtful paresis.

All but one of these sera agglutinated *Treponema pallidum*, and in the majority of instances in dilutions of 1:5 and higher. One serum caused well marked agglutination in dilutions as high as 1:80; the majority showed agglutination in dilutions varying from 1:5 to 1:40.

TABLE V.

Agglutination of Treponema pallidum by the Sera of Patients Suffering with Paresis.

Case No.	Diagnosis.	Wassermann reaction.				Agglutination.					
		Luetin.	C. H.	S.	A.	1:2	1:5	1:10	1:20	1:40	1:80
1	Paresis.....	−	+++−	+	++++	+	+	+	+	−	−
2	"	±	++++	++	++++	+	−	−	−	−	−
3	"	±	++++	±	++++	+	+	+	−	−	−
4	"	±	++++	++++	++++	+	+	−	−	−	−
5	"	−	++++	++++	++++	+	+	+	−	−	−
6	"	−	++++	+	++	+	+	+	+	+	−
7	"	−	++++	++++	++++	+	+	+	+	−	−
8	"	+	+++	−	−	+	+	+	+	+	−
9	"	−	+	+	+	+	+	+	+	+	−
10	"	+	++++	++++	++++	+	+	+	+	+	+
11	"	+	++++	++++	++++	+	+	+	−	−	−
12	"	−	±	−	−	+	+	+	+	−	−
13	"	−	++++	++++	++++	+	+	+	−	−	−
14	"	−	+++	+++	+++	+	+	+	−	−	−
15	"	−	++	++	++	−	−	−	−	−	−
16	"	+	++++	++++	++++	+	±	−	−	−	−
17	"	+	++++	++++	++++	+	+	+	+	+	−
18	"	−	++++	++++	++++	+	+	±	−	−	−
19	"	−	++++	++++	++++	+	+	+	−	−	−
20	"	−	++++	++++	++++	+	+	+	−	−	−
21	"	+	++++	++++	++++	+	+	+	+	+	−
22	" (doubtful)...	−	−	−	−	+	+	±	−	−	−

In Table VI are shown the results observed with a group of cases yielding positive Wassermann or luetin reactions or both and regarded clinically as being syphilitic.

None of the five cases regarded clinically as dementia præcox of different types gave positive Wassermann reactions, although all these cases yielded a papular or pustular luetin reaction, and in three of them there was well marked agglutination of *Treponema pallidum*.

The results of these agglutination tests with the sera of patients in the late stages of syphilis show that agglutinin is especially likely to be found in the highest percentages of cases and in largest amounts at this period of the disease.

TABLE VI.

Agglutination of Treponema pallidum by the Sera of Patients in the Tertiary Stage of Syphilis.

Case No.	Diagnosis.	Luetin skin test.*	Wassermann reaction.				Agglutination.					
			Luetin.	C. H.	S.	A.	1:2	1:5	1:10	1:20	1:40	1:80
1	Tabes dorsalis	++	−	++	−	−	+	+	+	+	−	−
2	Taboparesis	+	−	++++	++++	++++	+	−	−	−	−	−
3	"	+	−	++++	++++	++++	+	+	+	−	−	−
4	Cerebrospinal syphilis	++	++	++++	++++	++++	+	+	+	+	−	−
5	" "	≐	−	++++	++++	++++	+	−	−	−	−	−
6	Dementia præcox	++	−	−	−	−	+	+	≐	−	−	−
7	" "	+	−	−	−	−	+	+	+	−	−	−
8	" "	+	−	−	−	−	≐	−	−	−	−	−
9	" "	++	−	−	−	−	+	+	+	+	+	+
10	" "	+	−	−	−	−	≐	−	−	−	−	−
11	Depressive mania	+	−	+	−	−	+	+	+	+	−	−
12	" "	+	−	++	−	−	+	+	−	−	−	−
13	Aortic disease	0	+	++++	++++	++++	+	+	+	+	−	−
14	" aneurysm	0	−	++++	+	++	+	+	+	−	−	−
15	Gumma of brain	0	−	++++	++	+++	+	+	+	+	+	−

* ++ indicates pustular reaction; +, well defined papular reaction; ≐, slight papular reaction.

Agglutination and Complement Fixation.

An analysis of the results of agglutination and complement fixation tests with the sera of 54 persons yielding positive Wassermann or luetin reactions or both and regarded clinically as syphilitic, shows the following:

1. The Wassermann antibody seems to appear in the blood serum before demonstrable amounts of agglutinin.

2. The Wassermann antibody appears to reach a much higher degree of concentration in the blood serum.

3. The Wassermann antibody may be present while sufficient agglutinin to produce agglutination in dilutions of 1:5 and higher is absent, dilutions lower than 1:5 being unreliable because of the possibility of agglutination in a dilution of 1:2 by the sera of normal persons. Of the 54 sera from all stages of syphilis yielding positive

Wassermann reactions agglutination in dilutions of 1:5 and higher was absent in 14 sera or 26 per cent.

4. On the other hand, in 4 sera, or about 7 per cent of this series, demonstrable amounts of agglutinin were present and the Wassermann antibody was absent.

5. Of 37 patients regarded as being in the later stages of syphilis (Tables V and VI), 31 yielded positive Wassermann reactions and 8 positive and 3 doubtfully positive reactions with an antigen of luetin; all the sera yielding positive reactions with the antigen of luetin showed the presence of a demonstrable amount of agglutinin; on the other hand, a number of sera showing the presence of agglutinin did not absorb complement with the luetin antigen.

SUMMARY.

The results observed with this strain of *Treponema pallidum* may be stated as follows:

1. Normal human serum did not agglutinate this culture of *Treponema pallidum* in dilutions of 1:5 or higher; equal parts of treponema culture and normal serum (dilution 1:2) resulted in agglutination in about 50 per cent of the sera. With the strain of *Treponema pallidum* used in this study specific agglutination was not considered as having occurred unless observed in dilutions of 1:5 or higher.

2. The agglutinating power of the sera of non-syphilitic persons suffering with various infections was not higher than that of normal sera.

3. Traces of specific agglutinin for this culture of *Treponema pallidum* were found in the sera of persons late in the primary stage of syphilis.

4. In the secondary stage of syphilis about 58 per cent of sera showed the presence of a demonstrable amount of agglutinin in dilutions of 1:5 and higher; in no instance, however, was agglutination in evidence in dilutions higher than 1:20.

5. In tertiary and latent syphilis, mostly infections of the central nervous system, agglutination in dilutions of 1:5 and higher was found with about 84 per cent of sera. The highest concentration of *pallida* agglutinin in the blood serum was found in the later stages of syphilis.

6. The formation of agglutinin for culture *Treponema pallidum* in human syphilis is probably tardy and does not reach a state of high concentration.

7. There was no direct relation between the presence of the Wassermann antibody and agglutinin for this strain in the sera of syphilitics. The former appears in the body fluids earlier and is probably in higher concentration in all stages of syphilis; while agglutination is most likely to occur with Wassermann positive sera, it may be occasionally observed with sera yielding negative Wassermann reactions. Likewise strongly Wassermann positive sera may not contain demonstrable amounts of agglutinin.

8. It is probable that different strains of *pallida* vary in susceptibility to agglutination. For example, in the first investigation on agglutinins for *Treponema pallidum* by Kolmer, the sera of syphilitics in different stages of the disease did not cause agglutination of one of Noguchi's strains in dilutions of 1:20 and higher, whereas the strain used in the present study was frequently agglutinated in this dilution by sera from a similar group of patients. As shown by Zinsser, Hopkins, and McBurney, virulent *pallida* from human lesions resist agglutination to a remarkable degree.

9. While further studies are necessary to establish the practical value of agglutination in the diagnosis of human syphilis, we believe that with a suitable culture or cultures well marked agglutination of *Treponema pallidum* in dilutions of 1:5, or better 1:10 and higher, indicates *pallida* infection and may prove of value in the diagnosis of isolated cases, particularly in the later stages. Investigations bearing upon the relation of agglutinins for culture *pallida* to immunity in syphilis will be given in a separate communication (16).

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A STUDY OF THE PHYSIOLOGICAL ACTIVITY OF ADENOMATA OF THE THYROID GLAND, IN RELATION TO THEIR IODINE CONTENT, AS EVIDENCED BY FEEDING EXPERIMENTS ON TADPOLES.

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PLATES 38 TO 40.

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In 1912 and 1914 Gudernatsch[1] published the results of his experiments on feeding thyroid and other animal tissues to tadpoles. He concluded that thyroid has the "power to excite differentiation, but it lacks the power to cause growth." He used for these experiments fresh thyroid glands, the iodine content of which was not determined.

In 1914 Lenhart[2] carried out experiments along the same line as regards the thyroid, using for this purpose desiccated human, canine, sheep, and ox thyroids with iodine determinations on each specimen. He states that "the feeding of dried thyroid gland to tadpoles causes an early differentiation in proportion to the quantity fed or the percentage of iodine content of the gland used," and that thyroids with sufficiently low iodine content caused earlier differentiation but did not interfere materially with growth. "It all seems a question of dosage."

In Lenhart's work non-tumorous thyroid tissue was used. Since it has been established that the action of non-tumorous thyroid on tadpoles is dependent upon the iodine content, the following study was made to determine whether or not the so called tumors (adeno-

[1] Gudernatsch, J. F., Feeding Experiments on Tadpoles. I. The Influence of Specific Organs Given as Food on Growth and Differentiation, *Arch. Entwcklngsmechn. Organ.*, 1913, xxxv, 457; Feeding Experiments on Tadpoles. II. A Further Contribution to the Knowledge of Organs with Internal Secretion, *Am. J. Anat.*, 1913–14, xv, 431.

[2] Lenhart, C. H., The Influence upon Tadpoles of Feeding Desiccated Thyroid Gland in Variable Amounts and of Variable Iodine Contents, *J. Exp. Med.*, 1915, xxii, 739.

mata) of the thyroid, including carcinoma, would have the same action as non-tumorous thyroid and whether this action corresponds to the iodine content of the tumors.

For this purpose tadpoles (*Rana pipiens* and *Rana clamata*) were brought to the laboratory on May 8, 1915. They were fairly uniform in size (10 to 13 mm.) and age (estimated at about 1 week). The stock was kept in the laboratory in large granite basins and fed on fresh liver every day, the water (city tap) being changed once each day.

Thyroid Preparations.—Human thyroids were used in the experiments (Table I). Twenty-one specimens of desiccated thyroid were prepared from eighteen glands removed in Dr. Crile's clinic at Lakeside Hospital. Except in the case of No. 16 the portions of the fresh gland to be used were chopped fine, placed in a drying oven at 70°C. within 1 hour after removal, and allowed to remain in the desiccator from 4 to 6 days; they were then ground to a fine powder in a mortar and kept in sterile bottles until used. Iodine determinations were done on each specimen.[3] No. 16 was prepared from the same gland as No. 15 after the latter had been fixed in formalin. This was done to determine the effect of formalin fixation on the iodine content and the action on tadpoles.

The list of thyroids includes two simple adolescent colloid goiters (Nos. 12, 15, and 16) without tumors or complications; one unclassified gland (No. 3) which probably represents a stage between simple colloid goiter and diffuse colloid adenomatous goiter; and three diffuse colloid adenomatous goiters (Nos. 1, 2, 6, and 17) in which there was a diffuse colloid adenomatous change throughout the whole gland. There were also eleven glands with well encapsulated single or multiple adenomata of the fetal series in various stages of growth and differentiation from the almost pure fetal type to the well differentiated colloid or simple adenoma. Many of these showed some or all of the secondary degenerative changes frequently occurring in these tumors; namely, edema, recent and old hemorrhage, hyaline scars, and areas of calcification and cyst formation. This group includes in the order of their increasing differentiation Nos. 21, 19, 8, 4, 13, 20, 9, 10, 18,

[3] The iodine determinations were done by Dr. Marine.

TABLE I.

Desiccated Thyroid Preparations.

Thyroid No.	Specimen No.	Age.	Duration of goiter.	Clinical diagnosis.	Weight of specimen.	Pathological diagnosis.	Iodine per gm. of dried gland.
		yrs.	*yrs.*		*gm.*		*mg.*
1	11,714	57	20	Goiter, adenomatous.	700	Diffuse colloid adenomatous goiter.	0.22
2	11,714	57	20	" "	700	Diffuse colloid adenomatous goiter.	0.12
3	11,725	26	12	" " colloid, multiple.	735	Unclassified.	0.55
4	11,765	29	16	Goiter, adenomatous.	70	Hyperplastic intermediate adenoma.	0.69
5	11,818	30	18	" "	200	Intermediate adenoma, well differentiated.	1.31
6	11,819	55	31	" colloid.	225	Diffuse colloid adenomatous goiter.	0.43
7	11,821	34	7	" adenomatous.	173	Multiple intermediate adenoma, well differentiated.	0.85
8	11,825	47	15	" "	145	Intermediate adenoma.	0.06
9	11,836	44	14	" "	90	Multiple intermediate adenoma.	0.00 or trace.
10	11,836	44	14	" "	90	Multiple intermediate adenoma.	0.15
11	11,844	61	12	" multiple.	70	Multiple intermediate adenoma, well differentiated.	0.17
12	11,878	21	6	" colloid.	192	Colloid goiter, adolescent type.	0.58
13	11,886	47	40	" adenomatous.	14	Multiple intermediate adenoma.	1.23
14	11,898	58	1½	Adenoma.	273	Carcinoma; malignant adenoma.	0.00 or trace.
15	11,904	18	2	Colloid goiter. Appendicitis.	87	Colloid goiter, adolescent type.	1.00
16	11,904	18	2	Colloid goiter. Appendicitis.	87	Colloid goiter, adolescent type. (Formalin fixation.)	0.85
17	11,932	29	20	Colloid goiter.	710	Diffuse colloid adenomatous goiter.	0.18
18	11,933	55	12	Goiter, adenomatous.	545	Degenerating cystic intermediate adenoma.	0.07
19	11,938	31	16	" "	40	Intermediate adenoma.	0.06
20	11,939	44	⅓	" exophthalmic.	75	Multiple intermediate adenoma, well differentiated.	4.31
21	11,944	27	9	" adenomatous.	250	Fetal adenoma.	0.00 or trace

11, 7, and 5. There was one carcinoma (No. 14) of the malignant adenoma type.

In Nos. 1 and 2, 9 and 10, and 15 and 16, two preparations were made from each of three different glands. No. 20 was diagnosed as exophthalmic goiter, and No. 13 had been treated in the clinic 1 year previously for exophthalmic goiter. The others were diagnosed as simple colloid goiter or simple adenomatous goiter before operation. The carcinoma was not diagnosed as a malignant tumor before operation.

In the preparation of the specimens of adenomata the tumorous tissue was stripped from its capsule, care being taken not to include any of the surrounding non-tumorous thyroid tissue. In the case of the diffuse colloid adenomatous goiters, individual adenomatous nodules were shelled out with their capsules and the total mass was treated as described.

EXPERIMENTAL.

Series A.—May 11 to June 10, 1915 (duration, 31 days). The tadpoles were kept on a table in the center of the room so that light and temperature conditions were the same for all. Temperatures of the room and water were recorded each day.

Five tadpoles were placed in each of twenty-one granite dishes of about 500 cc. capacity, using 300 cc. of city tap water. Four basins with five tadpoles each were used as controls. Sample tadpoles were killed in formalin at the beginning of the experiment for standards of comparison. The tadpoles for experiment were fed 50 mg. of desiccated thyroids, Nos. 1 to 21, every 2nd day, alternating with fresh liver. The controls were fed fresh liver every 2nd day. The tadpoles in this series were not so uniform in size as was desired.

For this series merely the date of death in the different dishes may be given. The action of the different specimens of thyroid was essentially the same as to emaciation, growth, and differentiation as in Series B which is to be described in detail and will serve for both.

The following tabulation in conjunction with Fig. 1 gives a fair idea as to the time of death of the tadpoles and their condition at the time of death or at the termination of the experiment when they were killed in formalin.

Thyroid No.	Iodine.	Result.	Average time of death.
	mg.		*days*
20	4.31	All dead in 14 days.	12.4
5	1.31	" " " 16 "	13.8
13	1.23	" " " 21 "	12.8
15	1.00	" " " 22 "	21
16	0.85	" " " 24 "	22
7	0.85	" " " 19 "	16
4	0.69	" " " 28 "	23
12	0.58	" " " 24 "	22.4
3	0.55	Two " " 26 "; three lived.	31
6	0.43	All " " 29 "	22.8
1	0.22	All alive at end of 31 days.	
17	0.18	One dead in 25 days; four lived.	31
11	0.17	Two " " 29 "; three "	31
10	0.15	All alive at end of 31 days.	
2	0.12		
18	0.07		
8	0.06		
19	0.06	One dead in 30 days; four lived.	31
21	0.00	All but one, No. 14, lived 31 days.	
14	0.00		
9	0.00		
Controls.		Sixteen lived 31 days; no forelegs present. Four died showing no other differentiation than slight growth of the posterior leg buds.	

Definite changes were first noticed in this series on the 6th day in Nos. 20, 5, 13, 15, and 12. There was some wasting, beginning atrophy of the tail, and increased growth of the posterior leg buds. At the time of death all these, as well as Nos. 16, 6, 4, and 7, were smaller than the samples or the controls and showed much more differentiation than the controls.

As to the appearance of forelegs it is noteworthy that in the tadpoles in No. 20, four of which died in 12 days, all had visible left foreleg buds. In No. 5 all died between the 14th and 16th days and all showed foreleg buds. In No. 13 two died in 10 days with foreleg buds scarcely visible; forelegs were present on the other three. In No. 15 all died between the 20th and 22nd days; foreleg buds were present on two and doubtful on the other three.

Others in this series developed forelegs at different times corresponding closely in this and other respects with Series B. It is interesting to point out, however, that the large percentage of tadpoles living 31

days were being fed on thyroids with iodine contents of 0.22 mg. or less. It is also interesting that all these latter grew as much or more than the controls, and most of them showed a greater degree of differentiation.

Series B.—May 11 to June 28, 1915 (duration, 49 days). The procedure was the same as that in Series A, but smaller, white porcelain dishes with 150 cc. of water were used. The tadpoles in this series were quite uniform in size.

Table II and Fig. 2 give the results in this series, using the time of appearance of the first foreleg as an index to differentiation. This we think is justifiable because with the most active thyroid preparations used, and even in those tadpoles dying as early as 8 days this evidence of differentiation was present. It also offers a convenient

TABLE II.

Series B, Arranged According to Iodine Content.

Thyroid No.	Iodine content.	Time of appearance of first foreleg.	Average time of appearance of first foreleg.	Average time of death or killing.
	mg.	*days*	*days*	*days*
20	4.31	12	12.8	12.8
5	1.31	12	12.6	12.4
13	1.23	14	17.2	17.2
15	1.00	16	21.7	23.2
16	0.85	21	25.6	25.0
7	0.85	22	23.6	24.4
4	0.69	8	19.6	20.6
12	0.58	25	25.0	28.4
3	0.55	25	32.0	38.4
6	0.43	31	37.8	39.4
1	0.22	34	42.2	45.2
17	0.18	38	40.0	42.0
11	0.17	34	43.7	42.8
10	0.15	38	40.0	45.0
2	0.12	41	41.0	29.0
18	0.07	36	38.0	33.0
8	0.06	40	44.5	47.0
19	0.06	40	41.6	39.4
21	0.00 or trace.	40	46.4	48.2
14	0.00 " "	37	45.6	46.4
9	0.00 " "	40	43.2	45.0
Controls.....		45		

and useful method of comparison in determining the action of desiccated thyroid in causing differentiation of tadpoles. This is usually a fairly decisive indicator and may be readily recognized within a period of 24 hours of its occurrence.

In comparing Series A and B it is interesting to note that in Nos. 20, 5, 13, 15, 16, 7, 4, and 12, with iodine contents varying from 4.31 to 0.58 mg., all the tadpoles were dead in each series at the end of 31 days, the duration of Series A. In No. 3 (iodine content, 0.55

TABLE III.

Series B, Arranged According to Effects Observed on the 14th Day of the Experiment.

Thyroid No.	Iodine content.	Time of appearance of first foreleg.	Average time of appearance of first foreleg.	Average time of death or killing.
	mg.	*days*	*days*	*days*
20 5 13	4.31–1.23	12–14	12.6–17.2	12.4–17.2
15	1.00	16	21.7	23.2
16 7 4 12 3 6	0.85–0.43	21–31 (No. 4 in 8 days.)	19.6–37.8	24.4–39.4 (No. 4 in 20.6 days.)
1 17 11 10 2	0.22–0.12	34–41	40–43.7	42–45.2 (No. 2 in 29 days.)
18 8 19	0.07–0.06	36–40	38–44.5	33–47
21 14 9	Trace to 0.00	37–40	43.2–46.4	45–48.2
Controls		45		

mg.), two tadpoles in Series A and one in Series B were dead. In No. 6 (iodine content, 0.43 mg.) all were dead in Series A but only one in Series B. Below this level of iodine content (0.22 to 0.00 or trace) 91 per cent of each series were still living at the end of 31 days. It is evident then that a detailed description of the results in Series B will suffice for both within the time limits of 31 days. Series B is chosen for detailed study because of its longer duration (49 days).

The action of the desiccated thyroid was striking and consistent. On the 14th day of the experiment, before the iodine contents of the different thyroid preparations had been determined, the tadpoles were grouped according to the effect and the relative iodine contents of the different preparations predicted upon this basis. The prediction was correct with iodine contents from 4.31 down to 0.43 mg. in the order given in Table III. Below the level of 0.22 individual distinctions could not be made out with certainty, but all in this group were less affected than those receiving specimens with higher iodine contents.

Protocols.

Thyroid 20.—Changes were first observed in this dish on the 5th day; that is, after the second dose of thyroid. The change consisted of beginning kite-shaped appearance, prominence of the head, diminution in size or wasting, beginning atrophy of the tail, and increased growth of the hind leg buds. Three of these died on the 12th day and two on the 14th. All showed the characteristic changes and all at the time of death had visible left foreleg buds; all were smaller than the samples or controls. One became edematous.

Thyroid 5.—The changes, first noticed on the 5th day, were the same as in No. 20. Four died on the 12th day and one on the 14th, showing marked wasting and tail atrophy; three had a frog-shaped body and two were definitely kite-shaped. At the time of death left foreleg buds were visible on three and not observed on two; all were smaller than the samples or controls; two were somewhat edematous.

Thyroid 13.—The changes, first observed on the 6th day, were similar to those in Nos. 20 and 5. One died on the 14th day, two on the 16th, and two on the 20th. The left foreleg was present on each. One dying on the 20th day had both foreleg buds. All were smaller than the samples or controls; four were somewhat edematous.

Thyroid 15.—Changes similar to the above were observed on the 6th day. All showed marked wasting, tail atrophy, and considerable differentiation. These died on the 16th, 23rd, 24th, 25th, and 28th days, respectively. Two had left foreleg buds present at the time of death; the one dying on the 28th day had

both forelegs and the body was beginning to present the typical frog shape. One in this dish was lost.

Thyroid 16.—Changes were first observed on the 7th day. One died on the 21st day, 2 in 24 days, and 2 in 28 days. The left foreleg was present on three; not observed on two. All in this dish were quite edematous; all showed typical head changes and some were assuming frog-shaped bodies.

Thyroid 7.—Changes were first observed on the 7th day. Three died in 22 days, and two in 28 days. The left foreleg bud was present on all; all showed typical changes and were assuming frog bodies; slight edema in all.

Thyroid 4.—Slight change of shape was first observed on the 7th day. These tadpoles died in 8, 22, 23, 24, and 26 days. All were smaller than the controls; four were edematous. They showed various degrees of tail atrophy and were slightly kite-shaped. The three dying in 8, 24, and 26 days, respectively, had left foreleg buds. None were observed on the other two.

Thyroid 12.—Slight change of shape was first observed on the 7th day. One died in 26 days, two in 28, and two in 30. The first was highly edematous and showed no differentiation beyond the controls; no foreleg bud. The others were small and had frog-like bodies; the left foreleg bud was present on each; tail atrophy one-half to two-thirds.

Thyroid 3.—A slight change of shape was first observed on the 8th day. One died in 28 days, one in 36, two in 40, and one in 48. The first had a tadpole body, no tail atrophy, posterior leg buds about the same as the controls and no forelegs, and was highly edematous. The second had slight tail atrophy and the left foreleg bud through the skin; it was smaller than the control. The other three had frog-like bodies; both forelegs were present on each; tail atrophy one-third to two-thirds.

Thyroid 6.—In this dish it was doubtful whether definite changes could be made out on the 8th day. The tadpoles died in 31, 36, 39, 42, and 49 days. At the time of death all had left forelegs; the third and fourth had both forelegs, and on the fifth the right foreleg was present under the skin. Three had typical frog-shaped bodies and two were slightly frog-shaped.

Thyroid 1.—No change was observed up to the 8th day. Four died in 40, 43, 45, and 49 days, respectively. All were well differentiated frogs with almost complete atrophy of the tail. One which lived 49 days had a tadpole body and no forelegs. This is the first tadpole of the series fed on thyroid with iodine of 0.22 mg. or above which lived the 49 days of the experiment. Above this level of iodine content the tadpoles showed progressive degrees of wasting, and early differentiation in proportion to the iodine content. In none of these did the thyroid-fed tadpoles keep up with the controls as to growth (size), but of course they showed greater differentiation. Below this level of iodine content the thyroid-fed tadpoles grew as well as, and in many cases better than the controls (growth inversely proportional to the iodine content). At the same time there was a greater degree of differentiation among the low iodine-fed tadpoles as compared with the controls.

Thyroid 17.—In the frogs in this dish forelegs appeared in 38, 39, 40, 41, and 42 days. The first four developed into normal frogs with well developed and functioning fore- and hind legs and were killed in formalin on the 41st day. The last one developed into a normal frog and was killed on the 46th day.

Thyroid 11.—One died in 34 days, with beginning atrophy of the tail, slight changes about the head, and no forelegs. This one was smaller than the controls. The other four developed forelegs in 34, 39, 46, and 46 days, respectively. Two of these were killed in 42 days, and the last two died on the 48th day. All four were well developed frogs.

Thyroid 10.—Forelegs appeared in 38, 39, and 43 days on three. These developed into normal frogs; one died on the 40th day and two were killed on the 43rd and 46th days. The other two became highly edematous, showed no greater differentiation than the controls, and were killed on the 48th day.

Thyroid 2.—In this dish the tadpoles died in 11, 22, 34, 37, and 41 days. The first three maintained the tadpole body, had no atrophy of the tail, and developed no forelegs. Some of these were counted accidental deaths and not attributed to thyroid action. The fourth, dead on the 37th day, was becoming frog-shaped; no tail atrophy; no forelegs. The fifth was the only one that developed a foreleg (41st day). This one had a well developed frog-shaped body; tail atrophy about one-half.

Thyroid 18.—One of these was lost; two died in 15 and 35 days; and two lived 41 days and were killed in formalin. The first (15 days) showed little or no differentiation beyond the controls; no forelegs. The second (35 days) had well developed hind legs and was assuming a frog-shaped body; no forelegs or tail atrophy. The third and fourth developed forelegs in 36 and 40 days, respectively, and were killed on the 40th day. One of these was a well developed frog with almost complete atrophy of the tail; the other developed good fore- and hind legs, with little atrophy of the tail, and was highly edematous.

Thyroid 8.—Four developed forelegs in 40, 42, 48, and 48 days, respectively. One, which lived 49 days, had a tadpole body, no tail atrophy, and no forelegs. The first three developed into normal frogs with functioning fore- and hind legs. The fourth had both forelegs but a tadpole body and little atrophy of the tail. The fourth and fifth, both having a tadpole body, were larger than the controls.

Thyroid 19.—Two died in 28 and 35 days, respectively, without forelegs. Tail atrophy had begun, one having a tadpole body, the other becoming frog-shaped. Two developed forelegs in 40 days, became normal frogs, and were killed on the 43rd day. The last one developed forelegs on the 45th day, and when killed on the 48th day was fairly well differentiated.

Thyroid 21.—Forelegs appeared in 40, 46, 48, 49, and 49 days, respectively. The first developed into a normal frog and was killed on the 45th day. The other four lived 49 days; two of these had well developed frog-shaped bodies, tail atrophy one-third, and functioning fore- and hind legs; one had the left foreleg; and the last one had the left foreleg under the skin.

Thyroid 14.—Forelegs appeared in 37, 45, 48, 49, and 49 days, respectively. The first died in 37 days, with a tadpole body, slight changes about the head, and the left foreleg under the skin; it was about the size of the controls. The second developed into a normal frog with functioning fore- and hind legs. The third had a well developed frog body, tail atrophy one-third, and the left foreleg present. The fourth and fifth were becoming frog-shaped, had slight tail atrophy, and left forelegs under the skin.

Thyroid 9.—Four developed forelegs in 40, 40, 45, and 48 days, respectively. These were well differentiated, had functioning fore- and hind legs, and various degrees of tail atrophy. The fifth was killed on the 43rd day; highly edematous.

Controls.—There were four dishes with five tadpoles in each. These received fresh liver every 2nd day. The first one developed the left foreleg on the 45th day and died on the 48th day, a well differentiated frog. On the 49th day there were four dead, one with well developed hind legs and a left foreleg, also beginning tail atrophy; one with well developed hind legs and no forelegs; the other two were well preserved tadpoles with short posterior leg buds, no forelegs, and no tail atrophy. Fifteen lived for 49 days and were well preserved tadpoles.

Fig. 2 shows the condition of the tadpoles in this series at the time of death or termination of the experiment when they were killed in formalin. The different groups are arranged according to the decreasing iodine content with the controls and samples last. The individuals of each dish are arranged from left to right according to the time of death; *e.g.*, in No. 20 the first three from left to right died in 12 days and the fourth and fifth died in 14 days.

We have no satisfactory explanation for the peculiar edematous appearance of some of the tadpoles. This was observed in individuals in different dishes without regard to the iodine content and appeared in some of the controls.

Series C and D were for the purpose of determining the action of desiccated thyroid on a different variety of tadpoles and at varying ages of this species (*Rana catesbiana*).

Series C.—May 25 to June 10, 1915 (duration, 17 days). (Fig. 3.)

Tadpoles averaging 4 cm. in length were used in this series. Three were killed in formalin at the beginning of the experiment for comparison; three were used as controls and fed fresh liver every 2nd day; the experimental tadpoles were fed 50 mg. of Thyroids 20, 15, 12, 17, and 14 with iodine contents of 4.31, 1.0, 0.58, 0.18, and 0.0, or trace, every 2nd day, alternating with fresh liver. At the beginning of the experiment the tadpoles had an average length of 4 cm., just visible posterior leg buds, tadpole bodies, and no sign of forelegs.

The controls all lived 17 days and were killed in formalin. They had an aver-

age length of 4.3 cm., maintained the tadpole body, and showed slight increase in the posterior leg buds as compared with the samples.

Thyroid 14.—All lived 17 days and were killed in formalin, had an average length of 4.2 cm., tadpole bodies, slightly better growth of the posterior leg buds than the controls, and no forelegs.

Thyroid 17.—All these were lost.

Thyroid 12.—One was lost. One died in 12 days, showing marked wasting and tail atrophy; considerable growth of the hind legs; left foreleg bud present; beginning frog-shaped body; length 2.5 cm. The third lived 17 days and was killed in formalin. This one was 3 cm. in length; the body was becoming frog-shaped; left foreleg bud present; tail atrophy about one-third.

Thyroid 15.—Two died in 11 days. Each measured 2.3 cm.; there was marked atrophy of the tail; posterior leg buds 5 mm. long; left foreleg bud present on one and not on the other; heads frog-shaped. The third died in 12 days; length 2.2 cm.; left foreleg present.

Thyroid 20.—All died in 10 days, measuring 2, 2.2, and 2.2 cm., respectively. All showed marked tail atrophy; left foreleg present on each; bodies becoming fairly frog-like.

This series shows that there is no qualitative difference in the action of desiccated thyroid on the different varieties of tadpoles used.

Series D.—May 27 to June 7, 1915 (duration, 12 days). (Fig. 4.)

For this experiment two sets of tadpoles of different ages with three in each set were used. The individuals of each age were alike as to size and condition of development. The older ones averaged 8.5 cm. in length, had posterior leg buds slightly over 2 cm. long, and had no forelegs. The younger set averaged 6.5 cm. in length, had just visible posterior leg buds and no foreleg buds. One of each age was killed in formalin at the beginning of the experiment for comparison. Another of each age was used for control and fed fresh liver every 2nd day. A third one of each age was fed 50 mg. of Thyroid 20 every 2nd day, alternating with fresh liver.

Both forelegs were present on the older thyroid-fed tadpole on the 7th day; the left foreleg appeared on the corresponding control on the 9th day. There was marked atrophy of the tail of the thyroid-fed tadpoles. The younger control had no forelegs; and the posterior leg buds were 8 mm. long; it maintained a tadpole body. The younger thyroid-fed animal had a frog-shaped head and body; left foreleg present; posterior legs 15 mm. long. The older tadpoles at the end of the experiment showed about the same amount of development of fore- and hind legs on both the controls and the thyroid-fed animal. There was marked tail atrophy in the latter and practically none in the former. The older thyroid-fed tadpole had become a well developed frog, while the corresponding control had the same characteristics to a slighter degree.

Comparison of samples, controls, and Thyroid 20 in Series C and D shows that desiccated thyroid of high iodine content administered to tadpoles of the same variety at different ages and stages of development produces the same effect; namely, immediate cessation of growth, rapid metamorphosis as evidenced by atrophy of the tail, increased growth of the posterior legs, and the development of forelegs. The younger the tadpoles at the beginning of thyroid feeding, the smaller the metamorphosed frog, and *vice versa*.

DISCUSSION AND CONCLUSIONS.

It seems evident from the foregoing experiments that the so called tumors (adenomata) of the thyroid possess the property of taking up iodine and metabolizing it into the active combination in the same way that the non-tumorous thyroid tissue does, although not so readily nor to the same degree, and the action on tadpoles of feeding desiccated tumorous thyroid tissue does not differ qualitatively from feeding desiccated non-tumorous thyroid tissue. The action in either case depends upon the iodine (active iodine) content, and in the case of the adenomata bears no constant relation to the state of their growth or differentiation.

Examination of Tables II and III shows that in the main this is true. There are, however, certain discrepancies as to time of death, appearance of first forelegs, degree of emaciation, and rate of growth in certain dishes of the series, the action being not quite parallel to the iodine content. Some of these discrepancies may be explained in part by accidents of feeding, slight differences in size, age, and susceptibility of the different tadpoles receiving the same thyroid, and also by the variations in the amount of thyroid consumed by the different individuals in the same dish. Lenhart has shown that the action of the same thyroid varies with the quantity fed. Another important factor which has to be considered is the condition of the iodine itself. It was suspected at the time of these experiments that the iodine might be present in an active and an inactive form, but no satisfactory proof of this assumption, at the begin ing of these experiments, was at hand. Support of this point has been afforded by the work of Kendall on the isolation of the active principle of thyroid and the separation of the iodine into two fractions. Since the com-

pletion of our experiments Marine[4] has demonstrated by means of perfusion experiments *in vivo* and *in vitro* that iodine is rapidly taken up by the thyroid cells, and though the iodine increase in the perfused lobe may be 1,000 per cent in 2 hours as compared with the control lobe, yet the action on tadpoles is no greater. It then becomes an important question to determine the time required by the thyroid to take up inorganic iodine and manufacture it into the active thyroid principle.

It is known that iodine is rapidly taken up by the thyroid, and in man the iodine content of the thyroid is subject to greater variations than in animals on account of the prevalent therapeutic use of iodine and the iodides in goiter and other conditions; even the iodine used in preparing patients for operations would increase the iodine content of the thyroid in a short time, so that one might expect such variations in the action of a given thyroid preparation fed to tadpoles as appear in these experiments.

In this connection it is interesting to note (Table II) that Thyroid 20 with 4.31 mg. of iodine was only slightly more active than No. 5 with 1.31 mg. of iodine. Two possibilities have to be considered here. First, No. 20 may have active iodine slightly greater than 1.31 mg. and the balance present as inactive iodine. Second, No. 5 with 1.31 mg. of iodine might represent the maximum possible effect under the conditions of the experiment and a larger quantity of active thyroid iodine could produce no greater effect.

Of course with the lower iodine contents the variations in effects might well come within the limits of errors of observation. Also the percentage error would be greater in the iodine determinations, accidents of feeding, etc.

Our conclusions as to the effect of feeding desiccated thyroid to tadpoles agree in general with those of Lenhart. The action of the thyroid depends not upon a specific stimulus to differentiation but upon a stimulation of metabolism in general in proportion to the active iodine and the quantity consumed. High iodine contents produce

[4] Marine, D., Demonstration *in Vitro* of the Specific Affinity of Thyroid Cells for Iodin, *Proc. Soc. Exp. Biol. and Med.*, 1915, xii, 132. Marine, D., and Feiss, H. O., The Absorption of Potassium Iodid by Perfused Thyroid Glands and Some of the Factors Modifying It, *J. Pharm. and Exp. Therap.*, 1915, vii, 557.

rapid emaciation, at the same time resulting in differentiation even in tadpoles dying in 8 to 12 days. Low iodine contents result in differentiation at an earlier period than the controls. Tadpoles fed on thyroid with practically no iodine grow better than the controls, in this instance the thyroid acting simply as a food.

Finally, the interest that the results of these experiments may have in connection with the question of function in tumor tissue should be pointed out. To those who hold that tumor lacks the capacity for physiological function, the adenomata of the thyroid could not be consistently regarded as tumors. To those who hold physiological function as a possible property of tumor tissue, the adenomata might be regarded as tumors. Future studies might warrant a recognition of different grades or degrees of tumor. On this basis the fetal adenoma (very little differentiation) might represent a higher degree of tumor than the diffuse colloid or simple adenomatous thyroid in which the adenomatous nodules are present to a great extent throughout the whole gland and are well differentiated. It is certain that there are all grades and degrees of growth and differentiation in the life history of fetal adenomata of the thyroid, from the pure fetal, undifferentiated adenoma with little or no iodine to the simple or colloid adenoma, well differentiated and with varying amounts of iodine approaching that of normal thyroid.

EXPLANATION OF PLATES.

PLATE 38.

FIG. 1. Series A. The condition of the tadpoles at the time of death or after they had been killed in formalin, at the end of 31 days.

PLATE 39.

FIG. 2. Series B. The condition of tadpoles at the time of death or after they had been killed in formalin, at the end of 49 days.

PLATE 40.

FIG. 3. Series C. The effect of desiccated thyroid on *R. catesbiana* tadpoles as compared with the larvæ of *R. pipiens* and *R. clamata*.

FIG. 4. Series D. The effect of desiccated thyroid on tadpoles of different ages.

Series A.

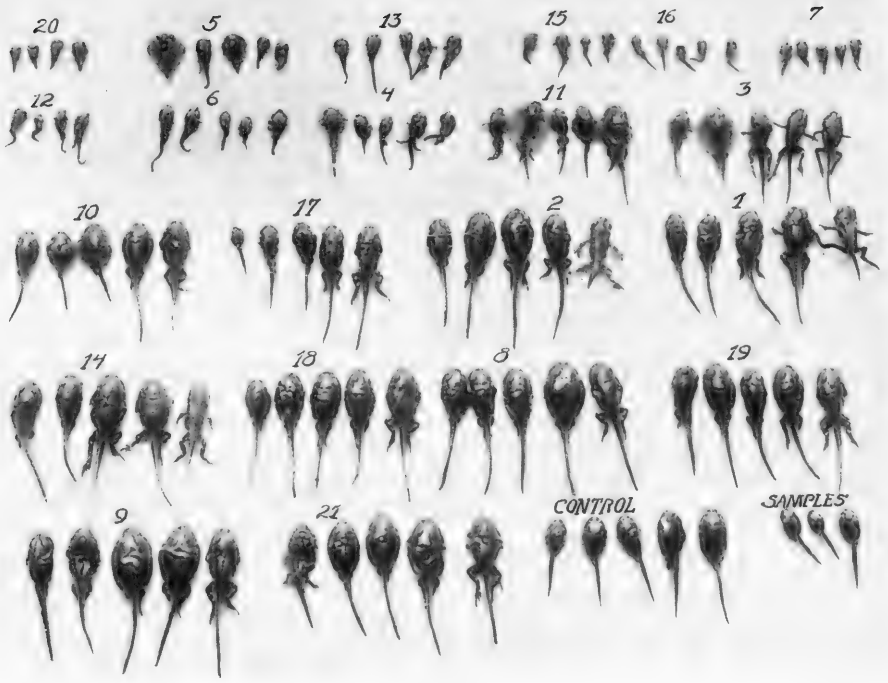

FIG. 1.

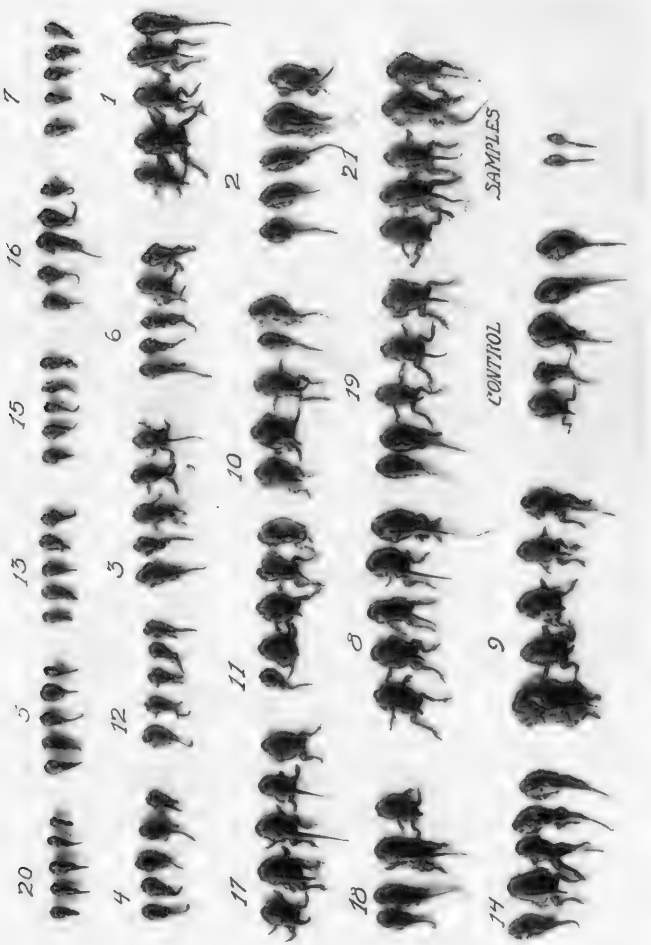

FIG. 2.

Series C.

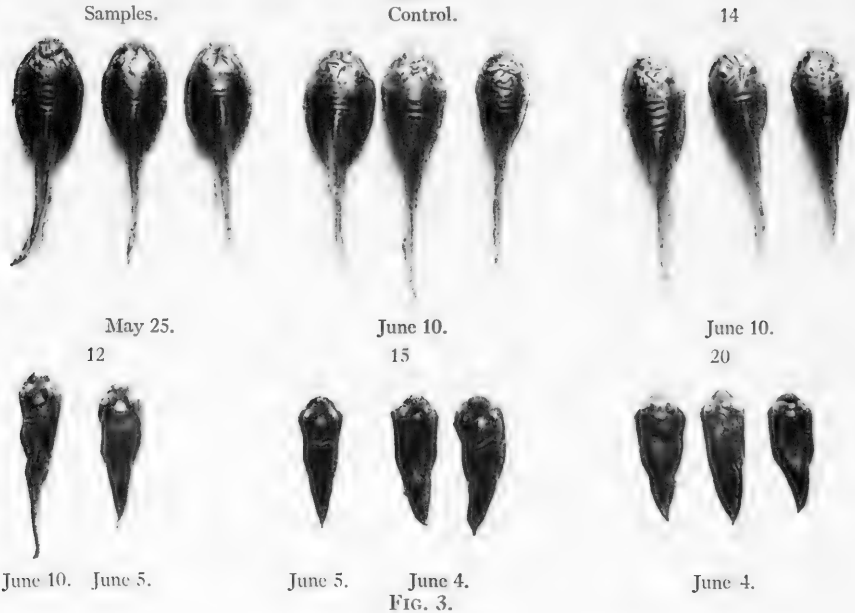

FIG. 3.

Series D.

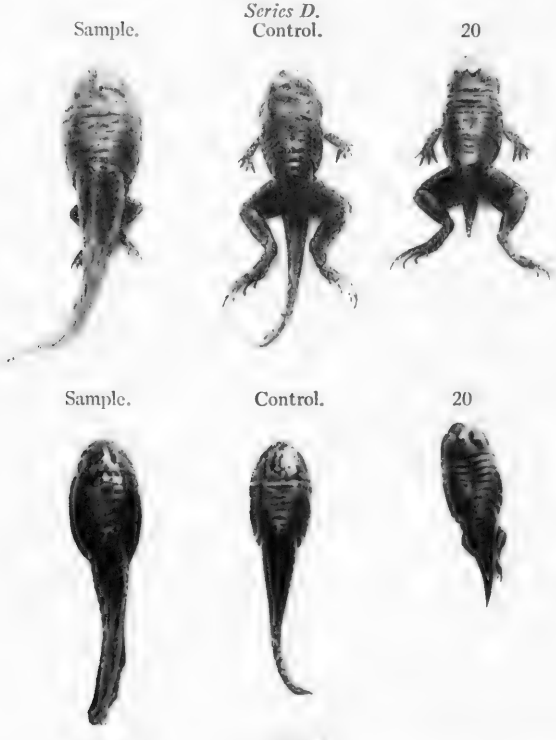

FIG. 4.

(Graham: Adenomata of the Thyroid Gland.)

SPONTANEOUS DIABETES IN A DOG.

By EDWARD B. KRUMBHAAR, M.D.

(*From the John Herr Musser Department of Research Medicine of the University of Pennsylvania, Philadelphia.*)

PLATE 41.

(Received for publication, June 21, 1916.)

Spontaneous diabetes occurring in the dog or other lower mammal is of interest on account of its rarity; and when associated with pancreatic lesions, it becomes a valuable link between the experimental diabetes of dogs and the spontaneous diabetes of man. While some pathologists believe that it occurs frequently in overfed, pet dogs, numerous published records of diabetes in animals are not readily found.

Fröhner (1) observed seven dogs in which persistent glycosuria and other clinical signs of diabetes were present, and quotes other cases observed by Schindelka, Eichhorn, Miller and Fettick (three each), and Gutzeit and Lienaux, Thiermesse, Schmidt, Wolff, Haltenhoff, Schulz and Strubing (one each). Eber (2) reported a series of twelve animals, and Lanfranchi (3) and Ferraro (4) each one; but all these occurred before Opie had demonstrated the now well known lesions of the islands of Langerhans. In four of Eber's five cases changes were noted in the pancreas; in one the organ was reduced by chronic inflammation to one-half its normal size; and in the other three, of normal size, numerous small yellowish abnormal specks were visible. It is noteworthy that the animals in all these cases were of advanced age and that the symptoms closely resembled those of human diabetes. Case reports of diabetes in other domestic animals are even rarer, though it has been found in the horse by Dieckerhoff (5), Preller (6), Kruger (7), Heiss (8), Rueff (8), and Perosino (9); in the cow by Girotti (10), Ingardi (10), and Darbas (9); and in the ape by LeBlanc (9). Preller's careful study of a case of spontaneous diabetes in a horse is the only instance in which associated pancreatic lesions have been investigated by modern methods of technique. At autopsy, an interacinous pancreatitis with extensive degeneration of the islands of Langerhans was found. The type of degeneration, however, is not stated and no study of the island cells with special granule stains was made. As to the rarity of diabetes in animals Fröhner states that the two cases first observed by him were the only ones in 40,000 examinations (incidence of 0.005 per cent).

From the clinical point of view it is noteworthy, in comparing the experimental diabetes of animals with that of man, that one of Fröhner's and one of Eber's dogs went into diabetic coma 5 and 3 days, respectively, before death. In two other animals of Eber's series, the diacetic acid test was positive on several occasions. As ketonuria has been observed by Allen in certain types of experimental diabetes in dogs, such cases form an additional link between the experimental and the spontaneous form of the disease.

Case Report.

A pet Airedale bitch, weight 19 kilos, age 9 years, after pregnancy and abortion in the spring of 1915, began to lose weight. The loss of weight was progressive, the animal was continually thirsty, ate ravenously of an unrestricted dietary, and, as in the case of Naunyn's depancreatized dogs, it was noticed that flies collected about her urine. Diabetes mellitus was suspected and it was found on collecting urine that a single drop completely reduced Fehling's solution (about 2 cc.).

Dr. A. E. Taylor kindly consented to study the animal's metabolism; but when she was placed in the strange conditions of a metabolism cage many difficulties arose which prevented detailed studies. It was found, however, that on a general diet the animal eliminated 60 gm. of sugar per day. When placed on 10 gm. of nitrogen daily (beef heart), the amount of sugar was reduced to 30 gm., a G : N ratio of 3. When fasting, the G : N ratio sank to 1.7, and the daily sugar output fell below 10 gm. At no time was there any ketonuria. It is possible that if the fast could have been continued longer the animal would have become sugar-free; but she was so obviously uncomfortable under laboratory conditions that the owner preferred to have her returned to her home where instructions that she was to be fed a carbohydrate-free diet were carefully carried out. Her clinical condition, however, steadily grew worse; sugar continued in the urine in large amounts, and after 3 months, cataracts developed in both eyes to the stage of total blindness. Abscesses and ulcers occurred on the legs and trunk, and she became greatly emaciated. A rapidly growing, freely movable, painless tumor appeared in the right side of the neck, and soon discharged watery and later bloody pus. She was chloroformed at the suggestion of her owner 6 months after the first symptoms had been noticed.

Autopsy.—Weight 10.9 kg. The pancreas was removed immediately after death and small sections (about 2 mm. thick) were placed at once in Zenker's, formalin, and sublimate-bichromate solutions. The pancreas was found to be large, rather soft, of normal color and shape, and without any signs of old or recent inflammation or hemorrhage in or about it. Weight 33.8 gm.; length 14.5 cm.; width at head 4 cm.; at middle 3 cm. The tissue cut easily, disclosing the normal lobular arrangement, without increase of fibrous tissue. Numerous minute, ocher-yellow points (1 mm. or less in diameter) were seen through the peritoneal covering, especially near the tail, but these were less noticeable on the cut surface. Otherwise the postmortem examination showed nothing worthy of note except a tumor, 83 gm. in weight, occupying the right side of the neck at the level of the thyroid cartilage but arising apparently in the thymus gland. A similar mass 2.5 cm. in diameter was found at the base of the heart.

Histology of the Pancreas.—The acini are well filled with zymogen, in some places so densely packed that these areas have an almost homogeneous reddish appearance (hematoxylin and eosin) with the acinous cells much obscured. In a few areas are collections of small round cells with localized increase of fibrous tissue, but these changes are not sufficiently numerous or extensive to be important. The blood vessels are normal. Striking abnormalities, however, are found in the islands of Langerhans. Not only are they scarce, but every island shows degeneration of one or more kinds (Fig. 1). The most obvious change, easily visible in almost every island, is the typical hydropic degeneration of one or more cells. Similar hydropic cells are prominent, either singly or in groups of three or four, in the interacinous spaces. These are probably miniature islands of Langerhans. As we did not have the necessary stain, the tissues fixed in sublimate-bichromate solution were sent to Mr. Walter B. Martin, of the Johns Hopkins Medical School, who kindly prepared sections stained with neutral azo-violet, to bring out the *alpha* and *beta* granule cells of the islands of Langerhans. Although the fixative did not penetrate for any great depth into the tissue, he was able to observe the changes in the islands fairly well, and reports as follows:

"The islets show complete degeneration of the *beta* cells, although remnants of these cells still are apparent in some of the islands as red staining areas. The *alpha* cells are also undergoing degeneration, but this has not progressed so far as in the others. The outline of the *alpha* cells can be made out, their nuclei are intact, and in a number of cases, the blue granulation is still quite distinct. Some of the islets are made up entirely of *alpha* cells undergoing degeneration."

Although some of the island cells have a hyaline appearance, the typical hyaline degeneration of Opie (11) and Weichselbaum (12) is not present. On the other hand, certain localized areas of fibrous tissue, especially those containing one or two isolated hydropic cells, show that in many cases whole islands have been replaced by fibrous tissue.

Histologic examination of the growth in the neck shows it to be a sarcomatous tumor of the thymus. The second tumor is a metastasis of the same in a lymph node. Thyroid, pituitary, adrenals, spleen, and liver show nothing of importance histologically, except extreme fatty change in the last named organ.

SUMMARY.

The picture is one of a true diabetes mellitus, first attracting attention after miscarriage. Although the G : N ratio sank from 3 : 1 on 10 gm. of nitrogen to 1.7 : 1 after 3 days' fasting, the disease progressed steadily in spite of a long continued carbohydrate-free diet. Together with the usual complications of diabetes, a malignant tumor of the thymus developed, so that after 5 months' observations a slow death was forestalled by chloroform. The most striking feature at autopsy was the large, apparently normal pancreas, which exhibited histologically marked changes in the islands of Langerhans, extreme hydropic degeneration and exhaustion of granules, involving both *alpha* and *beta* cells, but especially the latter, and replacement of some islands by fibrous tissue.

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11. Opie, E. L., *J. Exp. Med.*, 1900–01, v, 398, 527.
12. Weichselbaum, A., *Wien. klin. Woch.*, 1911, xxiv, 153.

EXPLANATION OF PLATE 41.

FIG. 1. Degenerated island of Langerhans. *a*, two cells showing extreme hydropic degeneration; *b*, normal island cells with *alpha* granules; *c*, two cells with *beta* granules that have escaped destruction. (In very few islands of this pancreas are these cells to be found.) *d*, island cells showing beginning hydropic degeneration; *e*, pancreatic acinus with lumen and apices of acinus cells filled with zymogen; *f*, fibrosis, involving most of the island; *g*, pancreatic acinus in which zymogen granules have failed to take the stain.

Fixation, sublimate-bichromate. Stain, Martin's azo-fuchsin-neutral violet.

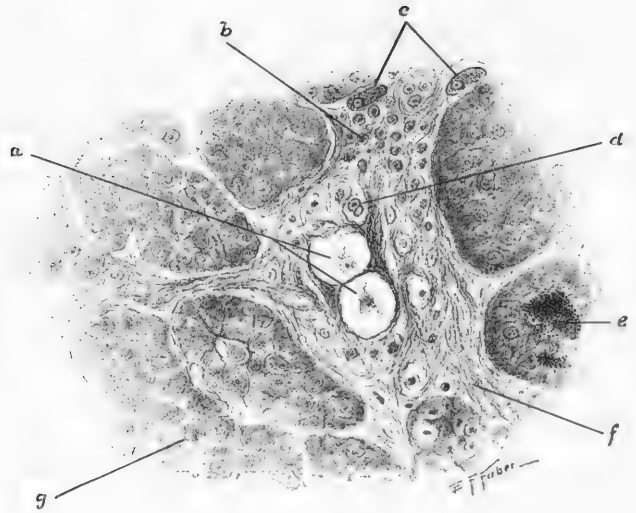

FIG. 1.

(Krumbhaar: Spontaneous Diabetes in a Dog.)

TECHNIQUE OF CULTIVATING HUMAN TISSUES IN VITRO.

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(Received for publication, June 22, 1916.)

In 1910 Carrel and Burrows[1] reported the successful cultivation *in vitro* of a human sarcoma. They used as a culture medium plasma from the patient from whom the tumor was removed, and observed an active migration of cells during several days' incubation. No subcultures were made. In subsequent attempts to cultivate human tissue Carrel[2] experienced considerable difficulty owing apparently to the liquefaction of the clotted plasma medium. He noted, as did also Lambert and Hanes,[3] Maccabruni,[4] and others, that within 24 hours a clear liquefaction zone appears around each tissue fragment, and that after a few days the entire fibrin clot becomes liquefied. Unless the cells wander out early they find no framework upon which to grow.

Losee and Ebeling[5, 6] attempted various modifications of human plasma with the object of preventing liquefaction, but were unsuccessful. However, by diluting the plasma with Ringer's solution, which seemed to delay digestion, and by making transfers of the tissue fragment to fresh plasma every 24 to 48 hours, they were able in a few instances to propagate human connective tissue cells obtained from fetuses through a number of subcultures, in one case as long as 60 days. They attributed their success in part to the addition of tissue extracts to the medium, as recommended by Carrel.[7] They emphasized the shortcomings of

[1] Carrel, A., and Burrows, M. T., Human Sarcoma Cultivated outside of the Body, *J. Am. Med. Assn.*, 1910, lv, 1732.

[2] Carrel, A., *J. Exp. Med.*, 1913, xviii, 287.

[3] Lambert, R. A., and Hanes, F. M., The Cultivation of Tissue in Plasma from Alien Species, *J. Exp. Med.*, 1911, xiv, 129.

[4] Maccabruni, F., Esperienzi di coltivazioni "in vitro" del cancro uterino umano, *Ann. ostet. e ginecol.*, 1914, xxxvi, pt. i, 57.

[5] Losee, J. R., and Ebeling, A. H., The Cultivation of Human Tissue *in Vitro*, *J. Exp. Med.*, 1914, xix, 593.

[6] Losee and Ebeling, The Cultivation of Human Sarcomatous Tissue *in Vitro*, *J. Exp. Med.*, 1914, xx, 140.

[7] Carrel, Artificial Activation of the Growth *in Vitro* of Connective Tissue, *J. Exp. Med.*, 1913, xvii, 14.

their technique and stated that they experienced many failures, for which the early rapid liquefaction of the fibrin was held responsible.

In our first efforts to cultivate human tissues in which unmodified human plasma was used as a culture medium, the results were practically the same as those of Carrel, and Losee and Ebeling. In only exceptional cases were we able to obtain satisfactory growths. There was regularly rapid digestion of the fibrin about the pieces of tissue, thus completely blocking cell migration. We therefore undertook to overcome this difficulty. It may be noted here that it is not the ability of human tissue to digest fibrin, but rather the susceptibility of human fibrin to digestion which is the basis of the trouble, for we found that human tissue did not liquefy the fibrin of foreign plasmas, while it was observed that human fibrin was readily digested by the ferments of practically every foreign tissue.

In the cultivation of the tissues of a number of lower animals,—rats, mice, guinea pigs, rabbits, dogs, cats, fowls, and pigeons,—using the plasma from these and other species, liquefaction was not infrequently observed, though never so regularly or extensively as in cultures containing human plasma. It was noted, moreover, that fowl and pigeon plasma were never liquefied, except in the presence of particular types of bacteria. Since earlier experiments had shown that tissues from certain of the lower animals could be cultivated in plasma from foreign species,[3] it occurred to us that this property of fowl and pigeon plasma to resist digestion might be utilized in growing human tissues. It seemed possible that chick fibrin might be made to serve as the framework of the culture medium with human serum or plasma added to supply the necessary nutritive substances. A medium was therefore prepared by mixing a small quantity of chick plasma with a considerably larger quantity of human serum. Various human tissues (lymph gland, spleen, skin, etc.) obtained at operation were put up in this mixed medium. No liquefaction was observed, and active progressive growth with numerous mitotic figures was seen in the majority of the preparations where soft friable tissue was used. Tissues removed at autopsy several hours after death were cultivated, the preparations showing in some instances a very active growth of connective tissue cells. It was not necessary

to make transfers to fresh plasma oftener than every 5 days. Continued propagation through subcultures was carried on for several months with as little difficulty as in the cultivation of similar tissues from lower animals. It may be noted that human tissues will grow in pure fowl plasma, but growth is not so active or so prolonged as in the modified medium containing human serum. Various proportions of fowl plasma and human serum were tested with the object of working out an optimum medium. A mixture of chick plasma, one part, and human serum, four parts, seemed to yield the best growth. The use of a relatively small amount of plasma has also an economic advantage in that human serum may obviously be obtained more easily and in larger quantity than fowl plasma. Furthermore, serum is easily preserved, whereas plasma sometimes clots after a few hours, even when kept cold in paraffined receptacles. It may be suggested that a still smaller proportion of plasma can be used since one part of plasma to twenty of serum will form a fairly firm clot.

The human serum used in these preliminary experiments came from a single individual. Autogenous plasma was not used since studies upon the cultivation of the tissues of lower animals had shown that plasma from the animal supplying the tissue was no better as a culture medium than plasma obtained from other members of the same species. For example, it has been found that the cells of transplantable mouse and rat tumors would grow quite as well in the plasma of rats and mice artificially or naturally immune to these tumors as in the plasma of tumor-bearing animals.[8] It seemed conceivable, however, that human tissues might prove more sensitive to individual variations in the culture medium. While studying this question it occurred to us that the influence of naturally existing iso-antibodies (agglutinins and hemolysins) upon growth *in vitro* might be investigated at the same time.

The interagglutinating reactions of human bloods, due, as Landsteiner and Leiner[9] and others have shown, to the existence of two

[8] Lambert and Hanes, A Study of Cancer Immunity by the Method of Cultivating Tissues outside the Body, *J. Exp. Med.*, 1911, xiii, 505.

[9] Landsteiner, K., and Leiner, K., Ueber Isolysine und Isoagglutinine im menschlichen Blut, *Centr. Bakteriol., Ite Abt., Orig.*, 1905, xxxviii, 548.

agglutinins and two agglutinogens in four different combinations[10] are well known. By simple agglutination tests any individual is found to fall into one of four groups depending on the power of his serum to agglutinate the blood of certain other persons, and on the susceptibility of his blood to agglutination; that is, on the presence or absence of one or both of the two agglutinins and agglutinogens.

For our experiments serum was obtained from individuals belonging to each of the four groups, and from the patients whose tissues were used for cultivation. The following protocol is illustrative of the results obtained.

Experiment 210.—An axillary lymph gland, removed at operation and showing microscopically changes characteristic of Hodgkin's disease, was placed in a sterile dish of cold salt solution. 1 hour later tissue cultures were put up in each of the following sera, to which chick plasma was added in the proportion of one part of plasma to four of serum: Group I, Group II, Group III, Group IV, and patient's serum (Group II). Ten cultures of each series were prepared and examined daily during the following week of incubation. Within 24 hours active migration of large mononuclear wandering cells and leukocytes was noted in practically every culture. On the 2nd and 3rd days a beginning outgrowth of connective tissue cells was observed, reaching a maximum activity about the 5th day. At no time could any difference in the five series be detected. The usual variations in individual cultures were seen, but the average extent of outgrowth was practically the same for each series. There was likewise no difference in the frequency of mitotic figures, which can be readily recognized by the practised observer in the living unstained preparations.

Other experiments in which tissues from Group I and Group III individuals were used gave the same result. While we were unable to secure any tissue from a person belonging to Group IV, as such

[10] It is assumed that there exist in human sera two agglutinins, *a* and *b*, and two corresponding agglutinogens, *A* and *B*. The blood of Group I possesses both agglutinins but no agglutinogens. It therefore agglutinates the blood of all other groups, but is not agglutinated by any serum. Group II possesses agglutinin *a* and agglutinogen *B*. It agglutinates blood of Group III and Group IV, and is agglutinated by members of Group I and Group III. Group III, the reciprocal of Group II, possessing agglutinin *b* and agglutinogen *A*, is agglutinated by Group I and Group II sera, and agglutinates the corpuscles of the third and fourth groups. Group IV contains no agglutinin but has both agglutinogens. Its serum therefore has no agglutinating power, but its corpuscles are agglutinated by the sera of all other groups.

people are rare, we feel justified in concluding that normally existing iso-antibodies in human blood do not influence in any way the growth of human tissues *in vitro*.

In addition to the question of culture medium we have been concerned with another problem in connection with the technique of cultivating human tissues *in vitro;* namely, the preservation of tissues after removal from the body. It is often not convenient to prepare cultures immediately upon the receipt of the tissue from the operating room or autopsy table. It is also desirable in many cases to carry out a number of experiments with the same tissue, not possible in a few hours' time. Furthermore, the duration of life of tissues in cold storage is a question of biological interest as well as of practical importance. In order to determine the period of survival under conditions of ordinary ice box preservation, cultures of the tissue were made on the day of removal, and at 2 day intervals thereafter, up to 12 days. A type experiment is described in the following protocol.

Experiment 320.—A piece of small tumor removed surgically from the region of the parotid gland (microscopically, a typical mixed tumor) was placed immediately in the ice box in a dish of physiological salt solution (0.8 per cent). Half an hour later the tumor, which was composed of soft friable tissue, rather gelatinous in places, was cut into small pieces. From some of these, cultures were made immediately. The remainder, covered with salt solution in a Petri dish, were returned to the ice box, an ordinary, small wooden structure with a lid, the temperature of which fluctuated between 10° and 15°C. The ten cultures put up on the day of operation grew well, showing after 2 to 3 days' incubation numerous large cells of irregular shape with frequent mitotic figures. Cultures made on the 2nd and 4th days of preservation, showed similar activity, though in the case of the tissue preserved for the longer time there was a longer latent period before migration was observed. Tissue kept for 6 days showed growth in the majority of preparations, though not so good as those put up on the 4th day. In the 8 day series only a few active cells were seen. Preparations of tissue kept for 10 and 12 days were all negative.

In another experiment in which a piece of lymph gland was used, no cells survived longer than 6 days. A glioma showed some growth after 8 days' preservation, but none after 10 days.

These experiments show that under very simple conditions of preservation human connective tissue cells and the cells of certain benign

tumors remain alive in the ice box for 6 to 8 days at least. 10 days probably represents the limit of survival. The period of survival is approximately the same as that determined for normal rat connective tissue, though somewhat shorter than that for embryonic chick tissues, which previous experiments have shown live 8 to 16 days in storage.[11] The optimum temperature for chick tissues was found to be about 6°C. The optimum temperature for preserving human tissues was not determined.

CONCLUSIONS.

1. Unmodified human plasma is not a satisfactory culture medium for human tissues owing to the susceptibility of human fibrin to digestion by tissue ferments. The necessary framework is thus destroyed before the cells begin to migrate. The difficulty can be overcome by adding to human plasma or serum a small quantity of fowl or pigeon plasma, the fibrin of which is highly resistant to digestion. Human tissues have been propagated in this medium for several months through subcultures, and growth *in vitro* can probably be maintained indefinitely.

2. Human tissues show no greater sensitiveness to changes in temperature and mechanical injury associated with preparation of cultures than those of lower animals. They may be preserved in an ordinary ice box at 10–15°C. as long as 6 or 8 days. Tissues obtained at operation give best results, but pieces of organs removed at autopsy 1 to 4 hours after death sometimes show active growth.

3. The presence of normally existing iso-antibodies (agglutinins and hemolysins) in human serum is without influence on the growth of human tissues *in vitro*. In other words, autogenous serum has no advantage in tissue cultures over homologous serum.

For the agglutination tests carried out in connection with the experiments reported, I am indebted to Miss M. P. Olmstead of the bacteriological laboratory of the Presbyterian Hospital.

[11] Lambert, R. A., The Influence of Temperature and Fluid Medium on the Survival of Embryonic Tissues *in Vitro*, *J. Exp. Med.*, 1913, xviii, 406.

A STUDY OF SERUM SALVARSANIZED IN VITRO.

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INTRODUCTION.

In a previous communication, Ellis and I[1] reported that the serum of patients who were treated intravenously with salvarsan or neosalvarsan had a definite spirocheticidal action. This action was increased by heating the serum at 56°C. for one-half hour. By an indirect method it was shown, first, that the heat destroyed some inhibitory substance in the serum, and, second, that it directly increased the action of the serum-salvarsan mixture. The beneficial effects of intraspinal injections of serum in the treatment of syphilis of the central nervous system have been attributed in part to this spirocheticidal substance. Because of the variable quantities of salvarsan in the serum of patients after intravenous injections of the drug, Ogilvie[2] has suggested a method for the preparation of salvarsanized serum in which a small quantity of faintly alkaline solution of salvarsan is added to serum, the mixture incubated and heated at 56°C. for one-half hour. The object of the present work was to study the spirocheticidal effect of serum salvarsanized *in vitro*.

Following our first communication, Stühmer[3] showed that the serum of salvarsan-treated rabbits had a trypanosomicidal action which was markedly increased by heating. He also showed that heating increased the intensity of the paradimethylamidobenzaldehyde color reaction for salvarsan, and that the intensity of the color reaction and the trypanosomicidal power were more or less parallel. However, by studying the serum daily for 10 days following the intravenous injection of salvarsan, he demonstrated that the trypanosomicidal action of heated serum persisted several days after the color reaction had disappeared. He[4] next showed that the trypanosomicidal action of the serum of salvarsan-treated rabbits persisted longer than that of neosalvarsan-treated rabbits, and that the serum of rabbits treated intramuscularly with salvarsan had very little action. Ehrlich

[1] Swift, H. F., and Ellis, A. W. M., *J. Exp. Med.*, 1913, xviii, 435.

[2] Ogilvie, H. S., *J. Am. Med. Assn.*, 1914, lxiii, 1936.

[3] Stühmer, A., *Münch. med. Woch.*, 1914, lxi, 745.

[4] Stühmer, *Münch. med. Woch.*, 1914, lxi, 1101.

suggested that the effect of heat was to break up a loose combination which had taken place between the salvarsan and some substance in the serum, thus setting free the salvarsan which acts on the trypanosomes or spirochetes; but Stühmer[5] attributed the increased action of heated serum to an increased oxidation. He finally studied the effect of oxidation and reduction on the salvarsanized rabbit serum and showed that oxidation did not increase the trypanosomicidal power of either unheated or heated serum, and also that reduction with weak sodium hydrosulphite solution did not decrease the power of the unheated serum. Reduction, however, did affect the heated serum by removing the increased trypanosomicidal action which had been produced by heating. In other words, the reduction caused the heated serum to act in the same manner as unheated serum. As Stühmer's experiments were not performed with diminishing dilutions of serum, but with serum obtained on successive days after the administration of salvarsan, the exact relation of heating, oxidation, and reduction are not entirely clear.

Methods.

In the present study a strain of *Spirochæta duttoni*, propagated in white mice, has been employed. The suspension for performing the test was prepared by bleeding a well infected mouse into warm normal saline solution, and diluting with normal saline solution until the required number of spirochetes per field was obtained. Equal quantities of this suspension and the dilution of serum to be tested were mixed thoroughly and incubated at 37°C. for 1 hour. 1 cc. of the mixture, representing 0.5 cc. of the spirochete suspension, was injected into mice intraperitoneally. The blood of the infected mice was examined daily with the dark-field microscope. The intensity of the subsequent infection is indicated by the average count of twenty fields, as shown in the protocols. The salvarsanized serum was prepared as follows: A slightly alkaline watery solution of salvarsan was diluted to 1:2,000 with 0.5 per cent saline solution. One part of this was added to nine parts of serum, making a dilution of 1:20,000, and from this the subsequent dilutions were made with normal serum, or normal salt solution, as the experimental conditions required.

EXPERIMENTAL.

The serum-salvarsan mixture prepared *in vitro* had a definite spirocheticidal action. This point was previously demonstrated by

[5] Stühmer, *Münch. med. Woch.*, 1914, lxi, 2338.

TABLE I.

Comparison of Serum Salvarsanized in Vitro with Serum of a Salvarsan-Treated Patient.

Mouse.	Dilution.	Salvarsan per cc. of serum.	Heated or unheated.	Day after injection.				
				1	2	3	4	5
	Salvarsan.	*mg.*						
A1	1:40,000	0.025	Unheated.	0	0	±w	0	0
A2	1:80,000	0.012	"	±w	+	+++	0	0
A3	1:160,000	0.006	"	±	+	+++	0	0
A4	1:320,000	0.003	"	±	++	+++	0	0
A5	1:640,000	0.0015	"	±w	++	+++	0	0
B1	1:40,000	0.025	Heated.	0	0	0	0	0
B2	1:80,000	0.012	"	0	0	0	0	0
B3	1:160,000	0.006	"	±w	+	+	0	0
B4	1:320,000	0.003	"	±	+	+++	0	0
B5	1:640,000	0.0015	"	+	+	+++	0	0
	Patient's serum.							
C1	1:1		Unheated.	±w	±	±w	0	0
D1	1:1		Heated.	±w	±	0	0	0
D2	1:2		"	±	++	±	0	0
E1	1:1		Unheated.	±	++	+	0	0
F1	1:1		Heated.	±w	++	±	0	0
F2	1:2		"	±	++	+	0	0
Control.								
S1	1:1			±w	++	+++	0	0

±w indicates between 1 and 19 spirochetes in 20 fields; ±, 1 per field; +, 2–10 per field; ++, 11–20 per field; +++, 20–50 per field; ++++, more than 50 per field.

Dilutions of salvarsan in normal serum were made, incubated, and each dilution divided into two portions; one of each portion was heated at 56°C. for one-half hour. A syphilitic patient weighing 71 kilos received 0.3 gm. of salvarsan intravenously (Sera C and D), and another weighing 68 kilos received 0.5 gm. (Sera E and F). Blood was taken from each after 1 hour, allowed to coagulate, centrifugalized, and the serum removed immediately. The serum from each was divided into two portions, one of which was heated at 56°C. for one-half hour, and then diluted as indicated. The spirochetal suspension, containing between six and eight spirochetes per field was mixed with the various sera; it was then incubated for 1 hour and injected into mice.

Gonder.[6] Heating the mixture at 56°C. for one-half hour increased this action. The effect of heating was studied in a series of seven experiments; four times the heating doubled the strength of the serum, twice it quadrupled it, and once increased it eightfold. In terms of absolute amounts of salvarsan in the heated serum, in three instances the spirocheticidal action was complete in a concentration of 0.025 mg., in three others of 0.012 mg., and once of 0.006 mg. per cc. of serum. This variation was probably due to the difference in the number of spirochetes in the suspension in the several experiments. In Experiment 1 (Table I) the heated serum salvarsanized *in vitro* was more effective in a dilution of 1:80,000 than the patient's serum, and the heating had less relative influence on the patient's serum. It should be noted here that the serum was removed from the clot within an hour of the time the patient was bled. In this respect this part of the experiment differs from those previously reported. In our earlier work the serum always stood over night before separation from the coagulum.

Although the spirocheticidal action of the unheated serum which was separated immediately was only slightly less than that which stood on the clot over night, a greater difference was noted between the two sera after they were heated. Heating increased the action of the serum which had been in contact with the clot over night much more than it did the serum removed from the clot immediately (Table II). Six different experiments were performed with the serum of patients obtained after salvarsan treatment. In four in which the serum was separated immediately, the increase in spirocheticidal action of heated serum was considerably less marked than in two in which the serum was removed from the clot 24 hours after the bleeding (Table VII, G and H).

After the demonstration of this point with the serum of salvarsan-treated patients, it seemed desirable to determine whether the serum from blood salvarsanized *in vitro* would act in the same manner. The results of this experiment are shown in Table III. Here the two unheated sera were again spirocheticidal in the same dilution, although Serum A in 1:2 dilution inhibited the infection 2 days

[6]Gonder, R., *Z. Immunitätsforsch., Orig.*, 1914, xxi, 309.

TABLE II.

Comparative Effect of Immediate Separation from Clot and Separation after Standing 24 Hours, with Serum from Salvarsan-Treated Patient.

Mouse.	Dilution of serum.	Heated or unheated.	Day after injection.			
			1	2	3	4
	Separated immediately.					
A1	1:1	Unheated.	+	+++	Dead.	−
A2	1:2	"	+	+++	±w	0
B1	1:1	Heated.	0	±	++	+++
B2	1:2	"	+	+	±	0
B3	1:4	"	±w	+	+	0
	Separated after 24 hrs.					
C1	1:1	Unheated.	+	++	+++	0
C2	1:2	"	+	+++	+	0
D1	1:1	Heated.	0	±w	+	±w
D2	1:2	"	0	±w	+	0
D3	1:4	"	±w	±w	+	±
Control.						
S1	Injected immediately.		+	++++	0	0

A patient weighing 65 kilos received 0.4 gm. of salvarsan intravenously. 50 cc. of blood were withdrawn after one-half hour, allowed to coagulate, centrifugalized, and 10 cc. of serum pipetted off. The remainder of the serum was allowed to stand on the clot over night, after which it was centrifugalized and pipetted off. Each serum was divided into two lots, and one of each heated at 56°C. for one-half hour. Dilutions of each series were made with normal saline; the spirochetal suspension containing from four to five spirochetes per field was added; it was then incubated for 1 hour and injected into mice.

longer than did Serum C in the same solution. When the two sera were heated, however, the one that was immediately removed from the clot was more potent than the one that stood on the clot over night. This is the opposite of what occurred with patient's serum. The explanation of this difference is not clear, but it is suggested that the salvarsan undergoes some change while circulating in the body which it does not undergo when it is mixed with blood in the test-tube. This may be due to the fact that in the body it is in contact with blood in the fluid condition for a longer period, hence oxidative conditions are more complete; or that contact with the tissues brings

TABLE III.

Comparative Effect of Immediate Separation from Clot and Separation after Standing 24 Hours with Serum from Blood Salvarsanized in Vitro.

Mouse.	Dilution of serum.	Heated or unheated.	Day after injection.						
			1	2	3	4	5	6	7
	Serum separated immediately.								
A1	1:1	Unheated.	0	Dead.*	−	−	−	−	−
A2	1:2	"	0	0	0	0	±	±w	±
A3	1:4	"	0	±	++	++	Lost.	−	−
A4	1:8	"	±w	±	++	0	0	0	0
B2	1:2	Heated.	0	0	0	0	0	0	0
B3	1:4	"	0	0	0	0	0	0	0
B4	1:8	"	0	±w	+	+++	0	0	0
B5	1:16	"	±w	+	++	+	0	0	0
	Serum separated after standing over night.								
C1	1:1	Unheated.	0	0	0	0	0	0	0
C2	1:2	"	0	0	±w	+	0	0	0
C3	1:4	"	0	±w	±w	0	0	0	0
C4	1:8	"	0	+	++	0	0	0	0
D2	1:2	Heated.	0	0	0	0	0	0	0
D3	1:4	"	0	0	±w	±	±w	0	0
D4	1:8	"	0	±w	+	±w	0	0	0
D5	1:16	"	±w	++	+++	+++	0	0	0

* Died from mouse typhoid infection.

2 cc. of a 1:2,000 dilution of salvarsan were mixed with 18 cc. of freshly drawn human blood. This was divided into two equal portions and allowed to coagulate. The first was then centrifugalized and the serum removed from the clot; the second was allowed to stand over night in the ice box, after which it was centrifugalized and the serum removed. Both lots of serum were divided into two portions, and one of each was heated at 56°C. for one-half hour. Dilutions of each of the four series were made with normal saline solution. The suspension of spirochetes, containing three to four spirochetes per field, was added; it was then incubated for 1 hour and injected into mice.

about some change in it which is not effected in the test-tube. From this point of view, at least, the use of serum of salvarsan-treated patients seems justified.

TABLE IV.

Comparison of Serum Salvarsanized in Vitro with Serum from Blood Salvarsanized in Vitro.

Mouse.	Dilution of serum.	Heated or unheated.	Day after injection.			
			1	2	3	4
	Salvarsanized serum.					
A1	1:1	Unheated.	0	0	0	0
A2	1:2	"	±w	±	+	0
A3	1:4	"	±	++	+	0
A4	1:8	"	+	++	+	Dead.
B1	1:1	Heated.	0	0	0	0
B2	1:2	"	0	0	0	0
B3	1:4	"	0	0	0	0
B4	1:8	"	±w	+	±	0
B5	1:16	"	±w	+	+	±w
	Serum from salvarsanized blood.					
C1	1:1	Unheated.	0	±w	±w	+
C2	1:2	"	0	±w	+	±w
C3	1:4	"	±w	++	+	0
C4	1:8	"	+	++	+	0
D1	1:1	Heated.	0	0	0	0
D2	1:2	"	0	0	0	0
D3	1:4	"	±w	±	++	0
D4	1:8	"	±w	+	++	0
D5	1:16	"	+	++	+++	+
Controls.						
S1	Injected immediately.		±w	++	Dead.	
S2	After 1 hr. incubation with normal sera.		+	++	"	

1 cc. of a 1:2,000 dilution of salvarsan was mixed with 9 cc. of normal human serum (making a dilution of 1:20,000), incubated for 1 hour, divided into two portions, A, unheated, and B, heated, at 56°C. for one-half hour. 1 cc. of the same salvarsan dilution was mixed with 9 cc. of freshly drawn blood, incubated for 1 hour, centrifugalized, and the serum pipetted off and divided into two portions, C, unheated, and D, heated the same as B. The various series were then diluted with normal saline solution; the spirochetal suspension, containing eight to ten spirochetes per field, was added; it was then incubated for 1 hour and injected into mice.

Experiment 4 (Table IV) was devised to determine whether a more potent serum resulted from the addition of salvarsan to serum or to blood. Both were prepared on the same day and tested within 2 hours of the time of preparation. In both the unheated and heated condition the serum prepared by the direct addition of salvarsan was more spirocheticidal than the serum from blood to which salvarsan had been added.

The demonstration that heating increased the spirocheticidal action of the salvarsan-serum mixture permitted a study of the effect of heat on each component of the mixture. This experiment confirmed by a direct method what was previously demonstrated by an indirect method, that part of the action of the heat was to remove some inhibitory substance from unheated serum. In Series C, Table V, the heating of the serum before the salvarsan was added resulted in producing a more active mixture than in Series A where the mixture was not subjected to heat. But the action of the mixture in Series C was not so strong as in Series B where the two were heated together. Series D demonstrated that part of the effect of the heat was directly upon the salvarsan, for although the heated salvarsan was combined with unheated serum, which contained inhibitory substances, the mixture was more spirocheticidal than the unheated mixture, but again not so active as the heated mixture. That the total result of the heat is due to a summation of these two effects is shown in Series E where the two components were combined after heating. This mixture was stronger than either C or D and had practically the same effect as in Series B where the two were heated together; the only exception noted was in Mouse E4 where there was a very slight infection on the 3rd day.

The relative effect of heat upon removal of inhibitory substance in the serum and upon increasing the spirocheticidal action of salvarsan was only slightly brought out in this experiment, where the infection of Mouse C4 was less than that of D4; but in two other similar experiments, in which the controls showed a more intense infection, the 1:80,000 mouse in the C series was completely protected, while the same mouse in the D series showed distinct infection. The effect of the heat seems, therefore, to be relatively greater in the removal of inhibitory substance from the serum than in directly increasing the activity of the salvarsan.

TABLE V.

Effect of Heat on Each Component of the Serum-Salvarsan Mixture.

Mouse.	Dilution of salvarsan.	Salvarsan per cc. of serum.	Heated or unheated.	Day after injection.				
				1	2	3	4	5
		mg.						
A1	1 : 20,000	0.05	Unheated.	0	0	0	0	0
A2	1 : 40,000	0.025	"	0	0	0	±w	±
A3	1 : 80,000	0.012	"	±w	+	+	0	0
A4	1 : 160,000	0.006	"	±w	+	±w	0	0
A5	1 : 320,000	0.003	"	+	+++	+	0	0
B1	1 : 20,000	0.05	Serum and salvarsan heated after mixing.	0	0	0	0	0
B2	1 : 40,000	0.025		0	0	0	0	0
B3	1 : 80,000	0.012		0	0	0	0	0
B4	1 : 160,000	0.006		0	0	0	0	0
B5	1 : 320,000	0.003		±	+	++	0	0
C1	1 : 20,000	0.05	Serum heated, salvarsan unheated.	0	0	0	0	0
C2	1 : 40,000	0.025		0	0	0	0	0
C3	1 : 80,000	0.012		0	0	0	0	0
C4	1 : 160,000	0.006		±w	±w	±w	0	0
C5	1 : 320,000	0.003		±	+	+++	±w	0
D1	1 : 20,000	0.05	Serum unheated, salvarsan heated.	0	0	0	0	0
D2	1 : 40,000	0.025		0	0	0	0	0
D3	1 : 80,000	0.012		0	0	0	0	0
D4	1 : 160,000	0.006		±w	±	+	0	0
D5	1 : 320,000	0.003		±w	+	0	0	0
E1	1 : 20,000	0.05	Serum and salvarsan heated separately before mixing.	0	0	0	0	0
E2	1 : 40,000	0.025		0	0	0	0	0
E3	1 : 80,000	0.012		0	0	0	0	0
E4	1 : 160,000	0.006		0	0	±w	0	0
E5	1 : 320,000	0.003		+	+	0	0	0
Controls.								
S1	Injected immediately.			+	++	+	0	0
S2	Incubated with normal serum.			+	++	0	0	0

The serum was from a patient with cured (?) syphilis. Wassermann reaction negative. Series A and B were prepared as in Table I. In Series C the serum was heated at 56°C. for one-half hour and then the salvarsan was added and dilutions were prepared. In Series D the salvarsan (1 : 2,000 dilution) was heated at 56°C. and then diluted with unheated serum. At the end of the period of heating the salvarsan was only very slightly turbid. Portions of the heated serum of Series C and heated salvarsan of Series D after heating were mixed in the usual way. The spirochetal suspension, containing between four and six spirochetes per field, was mixed with the various sera; it was then incubated for 1 hour and injected into mice.

TABLE VI.

Comparison of Salvarsanized Serum with Neosalvarsanized Serum.

Mouse.	Dilution.	Salvarsan per cc. of serum.	Heated or unheated.	Day after injection.			
				1	2	3	4
		mg.					
	Salvarsan.						
A1	1 : 20,000	0.05	Unheated.	0	0	0	0
A2	1 : 40,000	0.025	"	0	0	0	0
A3	1 : 80,000	0.012	"	±w	±	+	0
A4	1 : 160,000	0.006	"	±w	+	+	0
B1	1 : 20,000	0.05	Heated.	0	0	0	0
B2	1 : 40,000	0.025	"	0	0	0	0
B3	1 : 80,000	0.012	"	0	0	Dead.*	—
B4	1 : 160,000	0.006	"	0	±w	+	0
B5	1 : 320,000	0.003	"	±w	+	++	0
	Neosalvarsan.						
C1	1 : 20,000	0.05	Unheated.	0	0	0	0
C2	1 : 40,000	0.025	"	0	0	0	0
C3	1 : 80,000	0.012	"	0	0	0	0
C4	1 : 160,000	0.006	"	±w	±w	+	0
C5	1 : 320,000	0.003	"	±	++	+++	+
D1	1 : 20,000	0.05	Heated.	0	0	0	0
D2	1 : 40,000	0.025	"	0	0	0	0
D3	1 : 80,000	0.012	"	0	0	0	0
D4	1 : 160,000	0.006	"	0	0	0	0
D5	1 : 320,000	0.003	"	0	±w	++	0
Control.							
S1	Injected immediately.			+	++++	+	0
S2	After 1 hr. incubation with normal serum.			+	++++	0	0

* Died from mouse typhoid infection.

Dilutions of salvarsan were made in normal serum in the usual way. Similar dilutions of neosalvarsan in serum were prepared, 0.15 gm. of neosalvarsan being considered the equivalent of 0.1 gm. of salvarsan. Each dilution was divided into two portions, one of which was heated at 56°C. for one-half hour. The spirocheticidal suspension, containing six to eight spirochetes per field, was mixed with the sera; it was then incubated and injected into mice.

In comparing the serum withdrawn from salvarsan- and neosalvarsan-treated rabbits on successive days after treatment, Stühmer[4]

showed that the duration of the trypanosomicidal action of both was practically the same if the serum was unheated, and that there was but little difference between heated and unheated serum of the neosalvarsan-treated rabbits. The heated serum of salvarsan-treated rabbits, however, was trypanosomicidal several days longer than the same serum not heated. He concluded, therefore, that heating had less effect on neosalvarsanized serum. His method of experimentation did not lend itself to a comparative study of the effect of heating, because he used only one dilution of each serum. His results give rather the duration of trypanosomicidal effect after treatment, and the comparative rate of elimination of salvarsan and neosalvarsan. Experiment 6 (Table VI) showed that neosalvarsan is more active against *Spirochæta duttoni* than salvarsan, but that heating increases the action of both. In each instance the spirocheticidal action of the heated serum was double that of the unheated. Castelli[7] similarly showed that neosalvarsan was more active against *Spirochæta recurrentis* than salvarsan, the ratio being 1:600 to 1:400. The curative dose of both drugs was the same for *Spirochæta gallinarum*, while double the dose of neosalvarsan, compared with salvarsan, was required to cure syphilis in rabbits. Thus the comparative curative dose of the two drugs varies according to the microorganism tested. This experiment proves only that heat had the same relative effect on both salvarsan- and neosalvarsan-serum mixtures.

The proof that a mixture of salvarsan in normal serum had a definite spirocheticidal action suggested that this action might be enhanced if the salvarsan were mixed with the serum of a salvarsan-treated patient. The results of an experiment to determine this point are shown in Table VII. The lethal dose of the salvarsan in normal serum for the spirochete was 0.012 mg. The patient's serum (heated at 56°C.) was completely spirocheticidal in a dilution of 1:1. When half of this dilution was mixed with 0.006 mg. of salvarsan the mixture was completely spirocheticidal, and even when the same amount of salvarsan was mixed with a 1:4 dilution of serum only a slight infection of the mice occurred. Incidentally, the effect of heating salvarsan-treated patient's serum after it has stood 24 hours is well

[7] Castelli, G., *Z. Chemotherap., Orig.*, 1913, i, 321.

TABLE VII.

Effect of Adding Salvarsan to the Serum of a Salvarsan-Treated Patient.

Mouse.	Dilution of serum.	Salvarsan per cc. of serum.	Heated or unheated.	Day after injection.			
				1	2	3	4
		mg.					
	Normal.						
B3	1 : 1	0.012	Heated.	0	0	Dead.*	—
B4	1 : 1	0.006	"	0	±w	+	0
B5	1 : 1	0.003	"	±w	+	++	0
	Patient's serum.						
E1	1 : 1	0.006	"	0	0	0	0
E2	1 : 2	0.006	"	0	0	0	0
E3	1 : 4	0.006	"	±w	±w	±w	0
G1	1 : 1	None added.	"	0	0	0	0
G2	1 : 2	"	"	±	+	+	0
G3	1 : 4	"	"	+	+++	++	0
H1	1 : 1	"	Unheated.	±w	++	+++	0
H2	1 : 2	"	"	±	+++	++++	0
Control.							
S1	Injected immediately.			+	++++	+	0
S2	1 : 1	After 1 hr. incubation with normal serum.		+	++++	0	0

* Died from mouse typhoid infection.

Dilutions of salvarsan in normal serum were made in the usual way and heated (Series B). A patient with tabes, weighing 70 kilos, received 0.35 gm. of salvarsan intravenously. Blood was withdrawn one-half hour later and allowed to stand over night. It was then centrifugalized and the serum pipetted off, and three series of tubes, E, G, and H, were set up, and the serum was diluted with normal saline solution in Tubes 2 and 3. To each of the tubes of Series E, 0.006 mg. in 0.25 cc. of normal serum was added, after which Series E and G were heated at 56°C. for one-half hour. The spirochetal suspension, containing six to eight spirochetes per field, was added to each tube; it was then incubated for 1 hour and injected into mice.

brought out in the control series, G and H. This experiment seems to prove conclusively that the most potent spirocheticidal mixture with a given amount of salvarsan is to be expected from a combination of salvarsan with the serum of a salvarsan-treated patient, the

blood having been allowed to stand over night before the serum is separated.

DISCUSSION.

Following the introduction of intraspinal injection of serum in the treatment of patients with syphilis of the central nervous system, numerous workers attempted to inject intraspinally salvarsan or neosalvarsan in weak dilutions. When more than 1 mg. has been repeatedly injected several observers have noted symptoms of myelitis in the lower segment of the cord. Marinesco and Minea[8] have observed that neosalvarsan diluted with serum is less irritating than when diluted with normal salt solution. Fordyce[9] and Ogilvie,[2] who have had the greatest experience with the Ogilvie method of preparation of salvarsan serum, now advise that only a fraction of a milligram of salvarsan be mixed with the serum for intraspinal injection. Clinical evidence seems, therefore, to indicate that only small amounts of salvarsan or neosalvarsan can be safely injected into the subdural space. The main objection to subdural therapy of cerebrospinal syphilis has been that the serum injected contained such small amounts of salvarsan. Obviously it is necessary to keep the amount of any therapeutic agent below the injurious dose. Therefore, with the evidence at hand only small amounts of the drugs at our disposal can be used. Our object then must be to use them in the least injurious and most potent form. From the evidence brought forward in the experiments in this communication, this would be the addition of small amounts of salvarsan or neosalvarsan to the serum of salvarsan-treated patients. This serum preferably should be allowed to remain in contact with the clot over night, and should be heated after the addition of the salvarsan. Although this technique is complicated, it could be easily carried out in clinics where large numbers of patients are treated, or if the salvarsan is procurable in ampules containing small amounts, so that dilutions for addition of the drug to the serum might be economically prepared.

[8] Marinesco, G., and Minea, J., *Rev. neurol.*, 1914, xxii, 337.
[9] Fordyce, J. A., *J. Am. Med. Assn.*, 1914, lxiii, 552.

SUMMARY.

1. Addition of salvarsan to serum *in vitro* produces a spirocheticidal mixture which is increased in potency by heating.

2. The heated serum of salvarsan-treated patients is more spirocheticidal if it has been in contact with the clot over night than if it has been separated immediately after coagulation. This is not true with the serum from blood which has been salvarsanized *in vitro*.

3. The addition of salvarsan directly to serum produces a more potent mixture than results from the serum from blood to which salvarsan has been added in equivalent amounts.

4. The increase in activity of salvarsanized serum produced by heating to 56°C. is due in part to the removal of inhibitory substances in the serum and in part to a direct increase in spirocheticidal power in the heated salvarsan.

5. Both salvarsanized and neosalvarsanized serum are rendered more spirocheticidal by heating.

6. A more active spirocheticidal mixture is produced by mixing small amounts of salvarsan with the serum of a salvarsan-treated patient than by mixing the same amount with normal serum.

THE LATENT PERIOD IN THE GROWTH OF BACTERIA.

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The life cycle of a bacterial culture, as determined by estimations of the number of viable organisms present at various intervals, may be divided into four periods or phases, which, in the order of their appearance, may be designated: (1) latent period; (2) period of maximum rate of growth, or logarithmic period; (3) stationary period; (4) period of decline. These phases merge into one another without a sharp dividing line, and their duration varies with different species of organisms, and with the same organism under different conditions, such as temperature, nature of culture media, and still other factors.

By latent period or lag is meant the interval which elapses between the time of seeding and the time at which maximum rate of growth begins. During this time there may be slow growth, no growth, or an actual diminution of viable organisms. The present paper deals with the nature and significance of this phenomenon.

The logarithmic period, so called, is the phase of rapid growth during which the organisms are dividing regularly, so that their increase in a standard unit of time follows the law of geometric proportion, 2 bacteria giving rise to 4, 4 to 8, etc. If, during this period, the logarithms of the numbers of organisms found at varying intervals are plotted as ordinates, against time values as abscissæ, the logarithms fall upon an ascending oblique straight line (1).

This period is followed by the stationary period, in which the organisms cease to multiply at a maximum rate so that their increase in number becomes less and finally ceases; and, although they remain viable, the number present in a unit volume remains approximately constant for an appreciable length of time.

Finally the period of decline sets in, in which the number of living organisms begins to decrease, and at the expiration of several days or longer for certain bacteria living organisms cannot be demonstrated in the culture.

HISTORICAL.

The latent period in bacterial cultures was first recognized by Müller (2) in 1895 in the course of a study of the effect of high temperatures, simulating febrile conditions, upon the growth of the typhoid bacillus. Müller found that the latent period varied with the age of the culture used for seeding, being shorter for younger than for old cultures. He suggested that the latent period results from an alteration of the bacterial cells and that such bacteria transferred to a new favorable medium require time to recover from the injury sustained.

Hehewerth (3) ascertained that the duration of lag is less for *B. coli* than for *B. typhosus*, and shorter when the culture medium is one to which the organism has been accustomed.

Rahn (4) working with *B. fluorescens liquefaciens* studied the influence upon lag of the amount of bacteria used for seeding, and concluded that the larger the inoculum, the shorter the lag. Moreover, he reinoculated bacteria into bouillon from which the previous growth had been removed by filtration through infusorial earth or killed by heat, and found lag still present, although shorter than in fresh bouillon.

Barber (5) studied the rate of multiplication of single individuals of *B. coli* at different temperatures, and found that when three or four generations of bacteria had been formed in a culture, the individual members of that culture failed to show lag upon transplantation. Apparently he was the first to show conclusively that bacteria do not exhibit a latent period under these conditions.

Chick (6) found that *B. coli*, when grown in normal rabbit serum, shows lag, which varies with the temperature at which the organisms are grown, being longer when the temperature is below that of optimum growth.

The latent period has been extensively studied by Penfold (7), who has made an important contribution to the subject. He worked with *B. coli* and determined that with small seedings the lag is longer the smaller the inoculum, but with large ones variations in the size of the inoculum are without effect. He also confirmed Barber's observations, when he showed that subcultures made during the period of maximum rate of growth show no lag. In addition this author found that organisms subjected to a short application of cold during maximal rate of growth cease to multiply and that on reincubation they increase without lag. On the other hand, if the exposure to cold is long, lag appears on reincubation. Efforts to remove lag by washing the bacteria with saline were unsuccessful, as were efforts to demonstrate in the cultures the presence of heat-stable products which affect lag. Finally, he was able to show that organisms

remaining in the supernatant fluid after centrifugation of a culture continue to grow, but with a lag.

Measurement of Lag.—In estimating the rate of growth of bacteria it is convenient to speak in terms of their generation time, the formula for which was first worked out by Buchner, Longard, and Riedlin (8). If the increase always takes place by division of a single bacterium into two new bacterial cells, then, according to the law of geometric proportions $b = a \times 2n$, where b = the number of organisms obtained at the end of a given period of time T, a = the number of organisms present at the beginning of time T, and n = the number of generations which have occurred during the interval. Applying logarithms to this equation, the number of generations, or n, $= \frac{\log b - \log a}{\log 2}$. If there have been n generations in T time, obviously the generation time $G = \frac{T}{n}$.

Hence when multiplication is maximum, the generation time will be shortest, and when it ceases, or an actual decrease in a unit volume occurs, it will be infinity. It follows likewise that the generation time will vary for a single culture, depending upon the particular phase of growth during which observations are made. Consequently, estimations of generation time necessitate frequent observations upon the number of bacteria present; otherwise the results are not of great value.

The duration of lag is measured by the number of minutes or hours which intervene between the time at which the culture is seeded and the time at which maximum rate of growth commences. The onset of the maximum rate of growth corresponds to the minimum generation time.

EXPERIMENTAL.

Technique.—The bacteria employed in this study were *Diplococcus pneumoniæ*, *B. coli*, *B. prodigiosus*, and *B. fluorescens liquefaciens*. The strains of pneumococci used belonged to Types I and II[1] and were laboratory cultures which had been isolated from cases of lobar pneumonia several years previously, but subsequently had been passed through many white mice and transferred innumerable times on artificial media.[2]

The organisms were grown in beef infusion broth, made by extracting a pound of chopped beef with a liter of water over night on ice. The extract was filtered, made up to a concentration of 1 per cent peptone and 0.5 per cent sodium chloride, and boiled ½ hour. The reaction was adjusted to 0.2 per cent acid, using phenolphthalein as an indicator. It was then boiled again for 10 minutes, filtered

[1] See Dochez, A. R., and Gillespie, L. J., A Biologic Classification of Pneumococci by Means of Immunity Reactions, *J. Am. Med. Assn.*, 1913, lxi, 727.

[2] The cultures of *B. coli*, *B. prodigiosus*, and *B. fluorescens liquefaciens* were supplied by Dr. Kligler from the collections of the American Museum of Natural History.

twice through the same paper, and sterilized for 20 minutes on 3 successive days in an Arnold sterilizer. The readiness with which pneumococci grow in the different lots of media prepared in this manner has been found to vary. For this reason, to obtain comparable results with the pneumococcus it is necessary to have a large supply of a given lot of the medium. Results obtained with one lot of broth cannot be compared with those obtained with another lot.

The cultures were incubated in the dark in a water bath at approximately 37°C., in which the variation was always below 1°. Before seeding the broth was brought to 37°C. The number of organisms present in a unit volume was determined by diluting and plating, using 10 to 15 cc. of 1 per cent dextrose agar. The plate method was chosen instead of the direct method of Klein (9) which gives the total number of bacteria, dead and living, since the present study concerns itself especially with the number of living bacteria present in a culture. Dilutions were usually made in normal salt solution, but bouillon was employed when it was desired to observe growth of a few bacteria. Dilutions were carried out by tens, and so arranged that the dilution of the original culture was contained in 0.5 cc. of fluid, the unit volume. Dilutions were made with separate pipettes for each transfer of diluted culture.

During the period of maximum rate of growth of pneumococcus, chain formation is marked, and then diminishes as the age of the culture advances. Hence it might be urged that the latent period demonstrated by the plate method was apparent, rather than real, since a chain as well as a single bacterium may yield only one colony. The tendency of pneumococcus to form chains in fluid media constitutes a real objection to the use of this organism for accurate quantitative work, and renders difficult an estimation of the absolute duration of lag. However, we do not believe that the lag observed in broth culture can be explained on the basis of chain formation alone, for the plate method frequently shows that within the first 2 hours of incubation either no increase or an actual decrease in the number of viable organisms takes place. If the bacteria were multiplying rapidly during this time, although the plates showed only a slight increase or no increase at all, it would be necessary to assume that all or nearly all of the individual cells were forming chains, and that none of these chains were breaking up, an assumption which is scarcely warranted. Next, lag occurs in the case of all motile bacteria thus far investigated in which the question of chain formation can be excluded. Finally, studies which have been carried out in association with Mr. Glenn Cullen, and which will be reported in a subsequent paper, show that the increase in the hydrogen ion concentration of a broth culture of pneumococcus parallels the increase in the number of living bacteria during both the interval of latency and the period of maximum rate of growth, so that there is a lag in the growth of the culture and an equally long lag in the formation of hydrogen ions. The increase in hydrogen ion concentration observed in bacterial cultures indicates that metabolism is taking place, and hence is an expression of change in the medium produced by the agency of the bacteria themselves. It is improbable

that pneumococci carry on extensive metabolic processes independent of growth, since the bacterial cell is a unicellular organism and has a short term of life. Consequently, if, during the latent period no increase in the number of hydrogen ions is observed, it must be assumed that there is little or no metabolism taking place, and hence little or no increase in the number of bacteria.

Phases of Growth of a Bacterial Culture.

Experiment 1.—A flask containing 500 cc. of bouillon was inoculated with 0.2 cc. of an 18 hour broth culture of Pneumococcus Type II. Temperature of water bath 37°C. At frequent intervals a sample was removed for counting. The results are shown in Table I.

TABLE I.

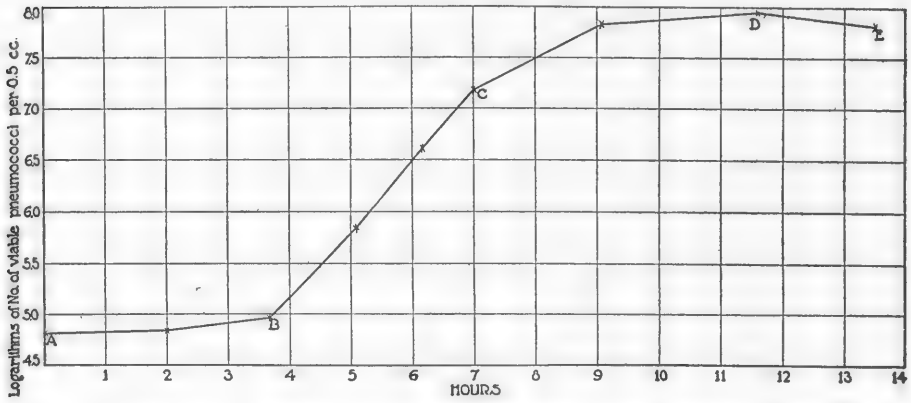
Life Cycle of a Culture of Pneumococcus Type II.

Time after seeding.	Viable bacteria per 0.5 cc.	Time.	No. of generations.	Generation time.
min.		*min.*		*min.*
0	65,000			
120	70,000	0–120	0.08	1,200.0
220	94,000	120–220	0.42	238.0
305	700,000	220–305	2.9	29.3
370	4,150,000	305–370	2.5	26.0
420	15,200,000	370–420	1.8	27.7
545	67,500,000	220–420	7.3	27.4
695	90,000,000	420–545	2.1	59.0
810	65,000,000	545–695	0.41	366.0
		695–810	0	

The table shows only a slight increase in the number of organisms at the end of 2 hours, and at the end of 3 hours and 40 minutes a single generation has not yet taken place. At the expiration of this period, a rapid increase in the number of organisms begins, which continues almost constant over a period of 200 minutes. These results are brought out more clearly by plotting a curve (Text-fig. 1), employing the time values as abscissæ and the logarithms of the number of organisms in a unit volume as ordinates.

The constant rate of increase during the period of maximum rate of growth is indicated by the fact that the logarithmic values of the number of organisms present fall upon a straight line between

the points *B* and *C*. The period of maximum rate of growth is succeeded by the so called stationary period, during which the growth is less rapid (*C* to *D* in the figure) as shown by the increase in the generation time, and finally the culture reaches the period of decline as indicated by the decrease in the number of viable organisms (from *D* onward). In this particular experiment this stage was reached about 12 hours after seeding. The experiment illustrates the four periods of growth—latent, maximal, stationary, and decline, described at the beginning of the paper. The present study relates to the nature of the first of these periods; namely, the latent period.



TEXT-FIG. 1. Growth curve of a bouillon culture of Pneumococcus Type II, at 37°C.

Latent Period.

Age of Inoculum and Latent Period.—Previous workers demonstrated the influence exerted by certain factors upon the occurrence and duration of this phase. The effect which the age of the culture used for inoculation exerts upon lag has an important bearing upon the nature of the phenomenon. Other investigators in this field all agree that the younger the culture used as inoculum, the shorter the lag. Although both Barber (5) and Penfold(7) have shown that lag is absent in subcultures made when the parent culture is growing rapidly, no attempt has been made to ascertain the particular time in the life cycle of a culture at which subcultures first display lag or

the length of time during which they fail to show it. The following experiments (Nos. 2 and 3), with pneumococcus and *Bacillus coli* as type organisms, show the behavior of subcultures made at frequent intervals during the various stages in the growth of the parent culture. The details of the experiments follow:

Experiment 2.—A flask containing 150 cc. of broth was inoculated with 0.3 cc. of a 24 hour broth culture of Pneumococcus Type I, stock strain, incubated in the water bath at 38°C., and subcultures were made one or more times during its various phases of growth. The growth of both parent culture and subcultures was observed at comparable intervals in each case by the plate method. The results are given in Tables II and III, and are represented in curves shown in Text-fig. 2.

TABLE II.

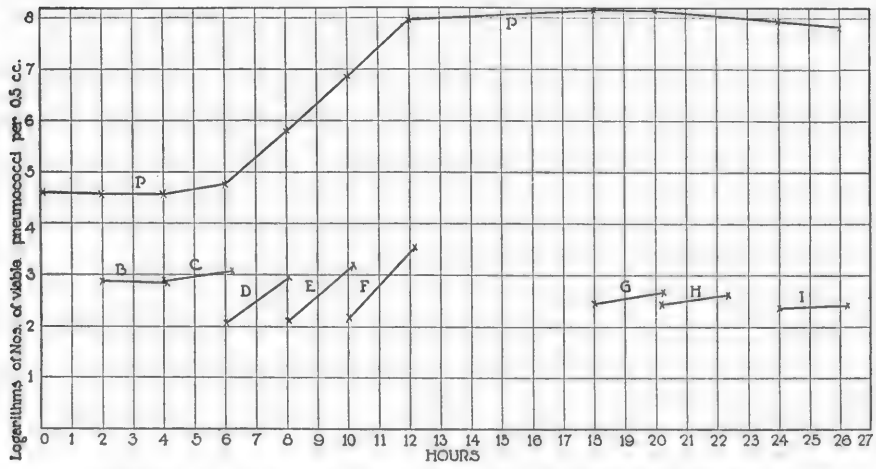
Comparison of Growth in Bouillon of Parent Culture and Subcultures of Pneumococcus Type I.

Parent culture.		Subcultures.		
Time after seeding.	Viable bacteria per 0.5 cc.	Subculture.	Time after seeding of parent culture.	Viable bacteria per 0.5 cc.
min.			*min.*	
0	41,000			
120	39,000	B	120	785
			245	713
240	38,000	C	240	766
			374	1,200
360	60,000	D	360	120
			485	898
480	625,000	E	480	127
			610	1,580
600	7,400,000	F	600	148
			730	3,490
720	95,000,000			
1,080	145,000,000	G	1,080	290
			1,215	483
1,200	136,000,000	H	1,210	273
			1,340	425
1,440	86,500,000	I	1,440	173
			1,575	263
1,560	65,000,000			

TABLE III.

Comparison of Growth of Parent Culture and Subcultures of Pneumococcus Type I, Expressed in Terms of Generation Times.

Parent culture.			Subcultures.			
Time after seeding.	No. of generations.	Generation time.	Subculture.	Time after seeding of parent culture.	No. of generations.	Generation time.
min.		*min.*		*min.*		*min.*
0– 120	0					
120– 240	0		B	120– 245	0	
240– 360	0.64	187.5	C	240– 374	0.65	206.1
360– 480	3.3	36.3	D	360– 485	2.9	43.1
480– 600	3.9	30.7	E	480– 610	3.6	36.1
600– 720	3.6	33.3	F	600– 730	4.5	28.8
720–1,080	0.6	600.0				
1,080–1,200	0		G	1,080–1,215	0.73	185.0
1,200–1,440	0		H	1,210–1,340	0.63	206.2
1,440–1,560	0		I	1,440–1,575	0.6	225.0



TEXT-FIG. 2. Growth of parent culture and subcultures of Pneumococcus Type I in bouillon at 38° C. *P* indicates parent cultures; *B* to *I* indicate subcultures.

Experiment 3.—A flask containing 50 cc. of bouillon was inoculated with two loops of a 24 hour bouillon culture of *B. coli*, and subcultures were made one or more times during the various phases of growth. The temperature of the water bath was 37°C. The growth of parent culture and subcultures was observed at comparable intervals in each case. The results are given in Tables IV and V and are represented in curves shown in Text-fig. 3.

TABLE IV.

Comparison of Growth in Bouillon of Parent Culture and Subcultures of Bacillus coli.

Parent culture.		Subcultures.		
Time after seeding.	Viable bacteria per 0.5 cc.	Subculture.	Time after seeding of parent culture.	Viable bacteria per 0.5 cc.
min.			*min.*	
0	20,000			
60	22,000	B	66	431
			129	1,390
120	64,500	C	120	120
			188	843
240	2,150,000	D	240	40
			309	349
			369	2,026
360	86,500,000	E	360	173
			426	614
525	485,000,000	F	525	107
			585	205
			645	1,257
705	970,000,000	G	705	194
			761	331
1,440	1,250,000,000	H	1,440	250
			1,508	378
			1,574	2,060
1,500	975,000,000			

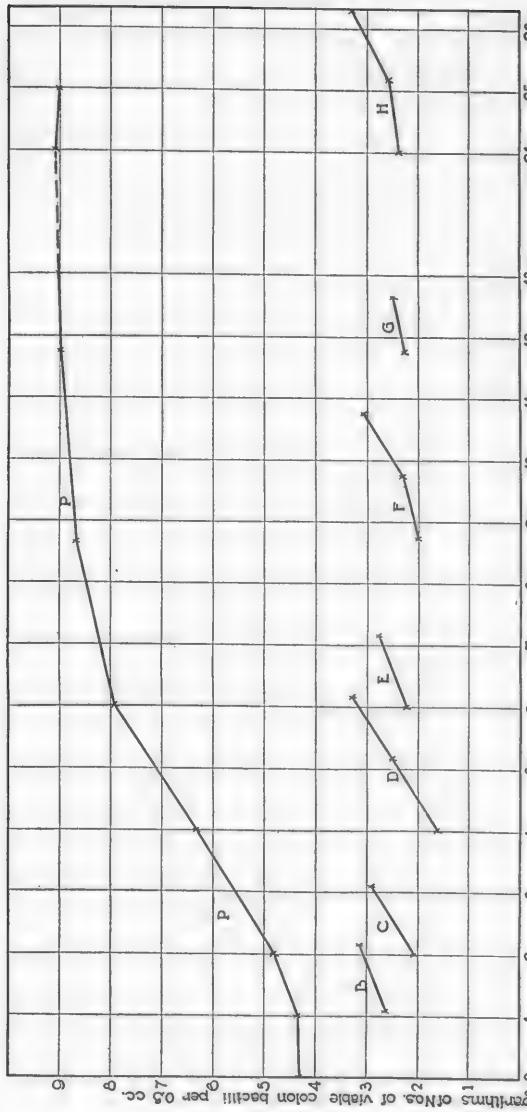
TABLE V.

Comparison of Growth of Parent Culture and Subcultures of Bacillus coli, Expressed in Terms of Generation Times.

Parent culture.			Subcultures.			
Time after seeding.	No. of generations.	Generation time.	Subculture.	Time after seeding of parent culture.	No. of generations.	Generation time.
min.		*min.*		*min.*		*min.*
0– 60	0.14	428.5				
60– 120	1.5	40.0	B	66– 129	1.6	39.3
120– 240	5.0	24.0	C	120– 188	2.8	24.2
240– 360	5.3	22.6	D	240– 369	5.6	23.0
360– 525	2.4	68.7	E	360– 426	1.8	36.6
525– 705	1.0	180.0	F	525– 585	0.93	64.5
705–1,440	0.36	2,041.6	G	705– 761	0.77	72.7
1,440–1,500	0		H	1,440–1,508	0.59	115.2

Experiments 2 and 3 show in both instances that if subcultures are made from the parent culture during the period of lag, the subcultures themselves show this same phenomenon. If, on the other hand, the subcultures are made while the parent culture is growing at a constant and maximum rate of growth, the subcultures show no latent period, but grow at approximately the same rate as that of the parent culture. Finally, subcultures made at a time when the parent culture is no longer growing at a maximum rate show a marked lag. This similarity in growth does not obtain if the subcultures are observed over a longer period of time, since after the latent period the subcultures repeat the entire cycle of the parent culture. Other experiments were carried out with *Bacillus fluorescens liquefaciens* and *Bacillus prodigiosus*, and identical results were obtained. They all show the importance of the relationship between the age of a culture and the presence or absence of lag in a subculture. Whether or not lag is present in a subculture depends upon the period in which the culture used for seeding happens to be at the time the subculture is made. That such was found to be the case with four different species of bacteria, motile as well as non-motile, suggests that the fact constitutes a principle applicable to all bacteria.

Occurrence of Lag in the Supernatant Fluid of Broth Culture.—The preceding experiments demonstrate that the behavior of bacteria,



TEXT-FIG. 3. Growth of parent culture and subcultures of *B. coli* in bouillon at 37°C. *P* indicates parent culture; *B* to *H* indicate subcultures.

when transferred to an environment favorable for growth, depends upon that particular period of the life cycle in which the culture happens to be at the time of transfer. The few bacteria remaining in the supernatant fluid after centrifugation of a culture at various phases in its life cycle were next studied to ascertain whether differences occurred in the behavior of the bacteria in the supernatant fluid depending upon the particular time at which the process of centrifugation was carried out.

Penfold (7) conducted one experiment with a 24 hour bouillon culture of *B. coli* and found that the bacteria which were left suspended in the supernatant fluid continued to grow on further incubation but with a definite lag. As no observations were made upon the growth of the culture which he used for centrifugation, it is impossible to say at what phase in its life cycle it happened to be at the time of centrifugation. As the supernatant fluid was kept on ice over night before incubation, a variable factor was introduced, since Penfold has shown that exposure to cold itself suffices to produce lag. No experiments seem to have been made with the organisms remaining in the supernatant fluid after centrifuging a culture during its period of maximum rate of growth.

Experiment 4.—A flask containing 500 cc. of bouillon was inoculated with 0.15 cc. of a 14 hour bouillon culture of Pneumococcus Type II, stock strain, and incubated in the water bath at 37.5°C. At three separate periods during the maximum growth of the culture, as indicated in Table VI, 50 cc. were removed and centrifuged at high speed for 30 minutes. Most of the bacteria were thrown down, leaving a clear supernatant fluid containing few organisms. The fluid was transferred to flasks which were incubated in the water bath and estimations of the number of viable organisms present were made at hourly intervals. The results are given in Table VI, and are further illustrated by curves in Text-fig. 4.

TABLE VI.

Growth of Pneumococcus Type II in Parent Culture and in Supernatant Fluids Obtained by Centrifuging at Stated Intervals.

Growth of Parent Culture.

Time after seeding.	Viable bacteria per 0.5 cc.	Time.	No. of generations.	Generation time.
min.		*min.*		*min.*
60	7,330			
120	11,000	60–120	0.58	103.4
180*	32,500	120–180	1.5	40.0
240	87,000	180–240	1.4	42.8
300*	331,000	240–300	1.9	31.5
360	887,000	300–360	1.4	42.8
420	4,850,000	360–420	2.4	25.0
480*	16,600,000	420–480	1.7	35.2
540	32,900,000	480–540	0.98	61.2
600	39,500,000	540–600	0.26	230.7
660	46,000,000	600–660	0.22	272.7
750	60,000,000	660–750	0.38	236.8

* At this point 50 cc. were removed from the culture and centrifuged at high speed for 30 minutes.

*Growth of Pneumococci in Supernatant A.**

Time after centrifuging.	Viable bacteria per 0.5 cc.	Time after centrifuging.	No. of generations.	Generation time.
min.		*min.*		*min.*
0	4,900			
120	111,000	0–120	4.5	26.6
240	1,300,000	120–240	3.5	34.2

* Obtained by centrifuging the parent culture 180 minutes after the initial seeding.

*Growth of Pneumococci in Supernatant B.**

Time after centrifuging.	Viable bacteria per 0.5 cc.	Time after centrifuging.	No. of generations.	Generation time.
min.		*min.*		*min.*
0	5,800			
120	132,000	0–120	4.5	26.6
240	610,000	120–240	2.2	54.5

* Obtained by centrifuging the parent culture 300 minutes after initial seeding.

TABLE VI—*Concluded.*

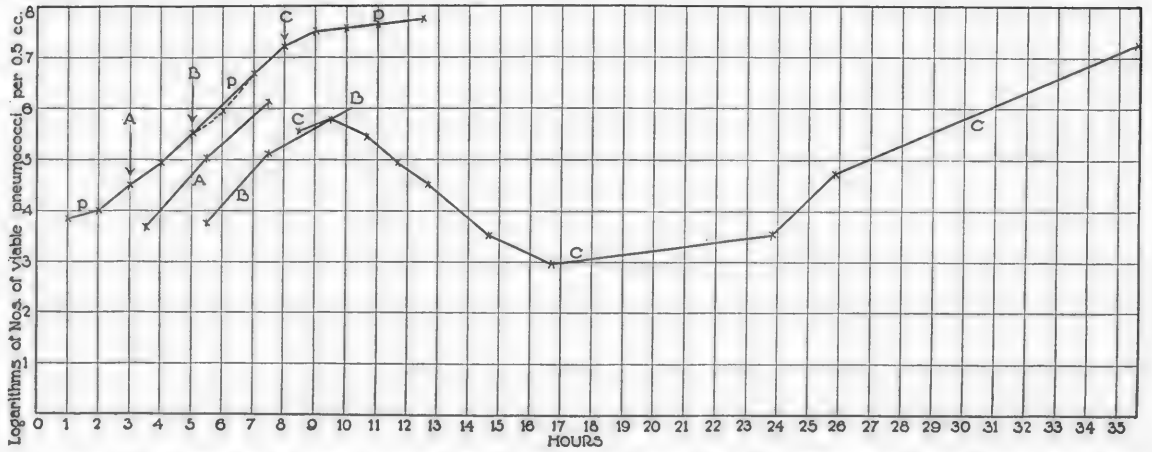
*Growth of Pneumococci in Supernatant C.**

Time after centrifuging.	Viable bacteria per 0.5 cc.	Time after centrifuging.	No. of generations.	Generation time.
min.		*min.*		*min.*
0	380,000			
60	630,000	0– 60	0.72	83.3
130	300,000	60– 130	0	
190	86,500	130– 190	0	
250	33,000	190– 250	0	
370	3,240	250– 370	0	
490	900	370– 490	0	
920	3,600	490– 920	2.0	215.0
1,040	55,000	920–1,040	3.9	30.7
1,630	17,000,000	1,040–1,630	8.2	71.9

* Obtained by centrifuging the parent culture 480 minutes after initial seeding.

Experiment 4 shows that during the early portion of the period of maximum rate of growth, the bacteria remaining in the supernatant fluid continue to increase in number at approximately the same rate as the organisms in the parent culture. If, however, the centrifugation is carried out toward the end of the period of maximum rate of growth, the viable bacteria in the supernatant fluid decrease in number over an appreciable interval of time (in this case 7 hours), then increase until finally, after 24 hours or more, there are present almost as many viable forms per unit volume as the parent culture attained. These results are illustrated graphically in Text-fig. 4, from which it is seen that the curves of growth of the bacteria in Supernatants A and B, obtained early in the period of maximum rate of growth, parallel the curve of growth of the parent culture; whereas the curve of Supernatant C, obtained at the end of the same period, does not parallel that of the parent culture, but shows, after the lapse of an hour, a fall and a subsequent rise. In other words, the bacteria in Supernatant C exhibit a marked lag. The conclusion to be drawn is that the presence or absence of bacterial lag in a supernatant fluid is dependent upon the age of the parent culture at the time centrifugation is carried out.

It is seen by reference to Text-fig. 4 that while the bacteria are



TEXT-FIG. 4. Growth of Pneumococcus Type II in parent culture and in supernatant fluids obtained by centrifuging at stated intervals. *P* indicates parent culture; *A*, *B*, and *C* indicate supernatant fluids. The arrows indicate the various times at which 50 cc. were removed from the parent culture and centrifuged.

dying the logarithms of the numbers of viable organisms, when plotted against time, fall upon an approximately straight line, indicating that the decrease in viable bacteria is a logarithmic one. It will be of interest to compare the behavior of pneumococci under such conditions with the course of destruction of bacteria when subjected to the action of disinfecting agents.

Krönig and Paul (10) showed that when anthrax spores are killed by disinfecting agents such as mercuric chloride the process is a gradual one. Madsen and Nyman (11) have confirmed these results, and have shown both from their own figures and from those of Krönig and Paul, that the process of disinfection takes place in accordance with a unimolecular reaction. Such a reaction is one in which a substance decomposes in such a manner that the amount undergoing decomposition in a unit of time bears a constant relation to the amount of substance present at the beginning of that interval of time. Chick (12) has shown that the same principle is applicable to the disinfection of anthrax spores and *B. paratyphosus* with phenol. The reaction is represented by the equation $\frac{dN}{dT} = KN$, which on integration yields $K = \frac{1}{t} \log \frac{N_1}{N_2}$ where K is the velocity constant, t is the interval of time between observations, N_1 the number of living bacteria present at the beginning of time t, and N_2 the number present at the end of time t. If the numbers of viable bacteria in a fluid medium decrease in accordance with this law, then the values determined for K should be constant when the values for N_1 and N_2 are substituted in the equation. Furthermore, the logarithms of the numbers of viable bacteria under such circumstances should fall on a descending oblique straight line when plotted against time. Text-fig. 4 shows that the bacteria in the supernatant fluid obtained at the end of the period of maximum rate of growth do decrease for several hours in such a manner that their logarithms fall upon an approximately straight line, which suggests that during this interval they are decreasing in accordance with the law of unimolecular reactions. On this account it seemed advisable to repeat Experiment 4 and obtain more frequent observations with a view of ascertaining whether the equation, which represents the course of such a reaction, can be strictly applied to the figures obtained experimentally.

Experiment 5.—A flask containing 500 cc. of bouillon was inoculated with 0.15 cc. of a 16 hour bouillon culture of Pneumococcus Type II, stock strain, and incubated in the water bath at 37.5°C. At two different intervals during the period of maximum rate of growth 50 cc. were removed and centrifuged at high speed for 30 minutes. The supernatant fluid was then removed to a flask and incubated further, and counts of both parent culture and supernatant fluids were made at frequent intervals. The results are given in Table VII and are further illustrated in the form of curves in Text-fig. 5.

TABLE VII.

Growth of Pneumococcus Type II in Parent Culture and in Supernatant Fluids Obtained by Centrifuging at Stated Intervals.

Growth of Parent Culture.

Time after seeding.	Viable bacteria per 0.5 cc.	Time.	No. of generations.	Generation time.
min.		*min.*		*min.*
0	13,100			
45	16,200	0– 45	0.3	150.0
185*	140,000	45– 185	3.1	45.1
300	915,000	185– 300	2.7	42.5
425*	11,500,000	300– 425	3.6	34.7
485	19,000,000	425– 485	0.7	85.7
560	39,100,000	485– 560	1.0	75.0
815	61,000,000	560– 815	0.6	425.0
1,220	100,000,000	815–1,220	0.7	578.5
1,575	113,000,000	1,220–1,575	0.17	2,088.2
1,880	80,000,000	1,575–1,880	0	

* At this point 50 cc. of culture were removed and centrifuged at high speed for 30 minutes.

*Growth of Pneumococci in Supernatant A.**

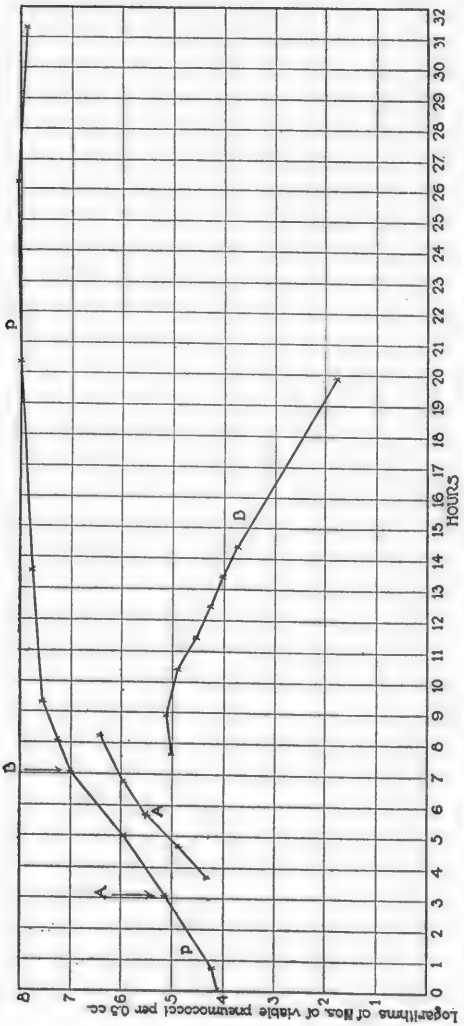
Time after centrifuging.	Viable bacteria per 0.5 cc.	Time after centrifuging.	No. of generations.	Generation time.
min.		*min.*		*min.*
0	21,300			
60	77,000	0– 60	1.8	33.3
120	335,000	60–120	2.1	28.5
185	885,000	120–185	1.4	46.4
275	2,650,000	185–275	1.5	60.0

* Obtained by centrifuging parent culture 185 minutes after initial seeding.

*Growth of Pneumococci in Supernatant B.**

Time after centrifuging.	Viable bacteria per 0.5 cc.	Time after centrifuging.	No. of generations.	Generation time.
min.		*min.*		*min.*
0	106,000			
75	132,500			
165	80,000			
225	35,000			
285	18,800			
345	10,900			
405	5,400			
735	60			
2,190	Flask cloudy; shows many pneumococci.			

* Obtained by centrifuging parent culture 425 minutes after initial seeding.



TEXT-FIG. 5. Growth of Pneumococcus Type II in parent culture and in supernatant fluids obtained by centrifuging at stated intervals. *P* indicates parent culture; *A* and *B* indicate supernatant fluids. The arrows indicate the various times at which 50 cc. were removed from the parent culture and centrifuged.

In Table VIII are shown the values of K obtained when the numbers of living bacteria in the second supernatant fluid are substituted in the equation $K = \frac{1}{t} \log \frac{N_1}{N_2}$. In this table are also shown the successive values for N_2 obtained by substituting for K its mean value, and for N_1 the number of bacteria found to be present when the organisms had begun to decrease. Opportunity is thus afforded to contrast the calculated theoretical values with those actually found by experimentation. This contrast is also illustrated in Text-fig. 6 in which the theoretical values are plotted in the form of a curve and the results determined by experimentation are designated as dots surrounded by circles.

TABLE VIII.

Application of the Behavior of Pneumococcus Type II in Supernatant B to the Law of Unimolecular Reactions.

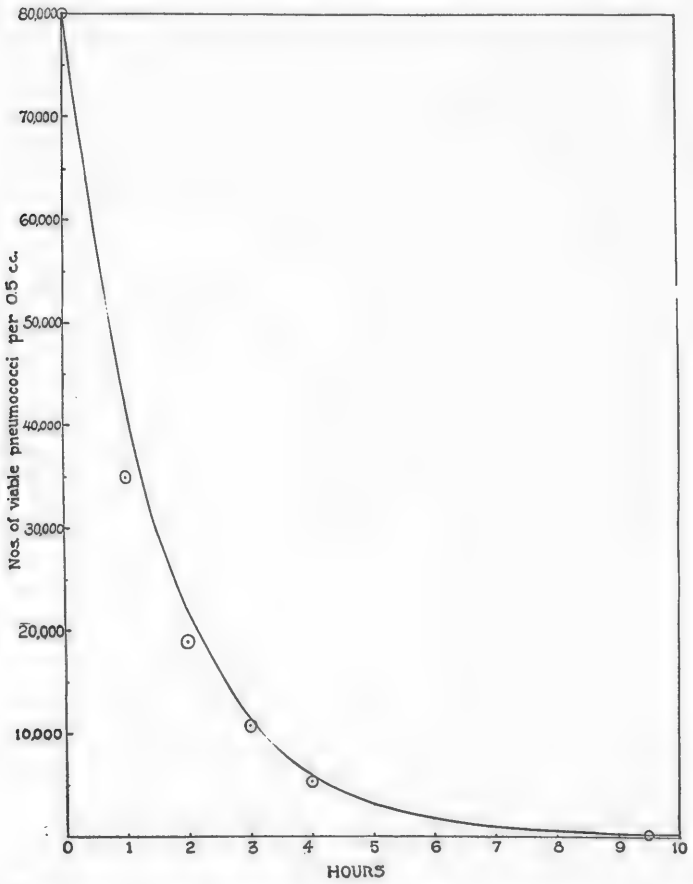
Time after seeding.	Viable bacteria per 0.5 cc.	Time.	Values of K calculated from equation $K = \frac{1}{t} \log \frac{N_1}{N_2}$ assuming $N_1 = 80{,}000$.	Successive values of N_2 assuming $N_1 = 80{,}000$ and $K = 0.0046$.
min.		*min.*		
625	80,000			
685	35,000	625– 685	0.0056	42,000
745	18,800	685– 745	0.0042	22,000
805	10,900	745– 805	0.0038	11,600
865	5,400	805– 865	0.005	6,100
1,195	60	865–1,195	0.003*	180
2,650	Flask cloudy; shows many pneumococci.			

Mean value of $K = 0.0046$

* The value for the constant K was not calculated when small numbers of bacteria were present, since Chick (12) has shown that in disinfection of *B. paratyphosus* when the culture has reached this stage the velocity of the reaction is slightly greater than one would expect, and the formula cannot be strictly applied.

Experiment 5 confirms Experiment 4 in that it shows that the pneumococci in the supernatant fluid at the beginning of the period of maximum rate of growth continue to multiply at the same rate as those in the parent culture, whereas the bacteria in the supernatant fluid obtained at the end of the period of maximum rate of growth do not continue to grow as do those in the parent culture, but exhibit a marked lag. This experiment shows also, as suggested by Experi-

ment 4, that the bacteria in the second supernatant fluid decrease in number in accordance with the law of unimolecular reactions, since the values obtained for K are quite constant, considering the experi-



TEXT-FIG. 6. Curve showing that the decrease of pneumococci in the supernatant fluid of a broth culture, centrifuged at the end of the period of maximum rate of growth, follows the law of unimolecular reactions. The solid line represents the curve of the calculated values; the dots surrounded by circles represent the values found by experimentation.

mental error necessarily introduced in counting. The closeness with which the course of the destruction of the bacteria follows that of a unimolecular reaction is illustrated by Text-fig. 6 in which the numbers of living bacteria found by experiment fall quite close to or upon the curve which represents the course of such a reaction. It may be said, then, that most of the pneumococci remaining in the supernatant fluid after centrifugation of a culture at the end of the period of maximum rate of growth die within a limited period of time in the course of which they follow the same law as do other bacteria when subjected to the action of disinfecting agents.

The fact that such a close parallelism exists between the behavior of bacteria subjected to the action of disinfectants on the one hand, and the behavior of the pneumococci in the supernatant fluid of broth cultures on the other, suggests that there are present in the supernatant fluid injurious substances that destroy large numbers of the bacteria and arrest the growth of still others. These hypothetical substances must be the products of bacterial growth and demonstrable in every broth culture of pneumococcus. But since the supernatant fluid does not become sterile and ultimately growth is resumed, it follows that either these injurious substances are destroyed or have their effect neutralized, or that the bacteria become adapted to them.

If pneumococci, when grown in broth, form substances inhibitory to their growth, it might be expected that these substances would be present in greatest concentration when the culture had reached the summit of growth. If, however, such substances are unstable on standing at 37°C. as suggested by Experiments 4 and 5, then when a broth culture of pneumococcus is incubated for some period beyond that of maximum growth, they should no longer be present in as great a concentration as that existing at the time when the culture was at its summit. Consequently, if the fluid portion of a culture which has undergone prolonged incubation is reinoculated with the same strain of bacteria, at least partial growth should result, provided the nutritive elements of the medium have not been exhausted. The next experiment was carried out to determine this point.

Experiment 6.—A flask containing 500 cc. of bouillon was inoculated with a culture of Pneumococcus Type II, stock strain, and incubated in the water bath at 37°C. for 3 days. Previous experiments (Nos. 1 and 2) have shown that at this time the culture has passed the summit of its growth, and that the bacteria are dying or are nearly all dead. The culture was then centrifuged until a clear supernatant fluid was obtained and this fluid was removed by a pipette to another container. Cultural tests showed only a few viable bacteria. These, however, died off rapidly on further incubation and at the end of 24 hours the fluid was found to be sterile. 50 cc. of this sterile fluid, representing the supernatant of a 4 day culture of pneumococcus, were inoculated with 1 cc. of a culture of the same strain which itself was in the period of maximum rate of growth (7½ hours old). 50 cc. of unused bouillon from the same original lot were inoculated with a like amount of the same culture, thus constituting a control. Both flasks were incubated at 37°C. in the water bath and counts made at frequent intervals. The results are given in Table IX.

TABLE IX.

Comparison of Growth of Pneumococcus Type II in the Supernatant Fluid of a 4 Day Culture of Pneumococcus Type II and in Unused Broth.

Growth in supernatant fluid of 4 day culture.					Growth in unused broth.			
Time after seeding.	Viable bacteria per 0.5 cc.	Time.	No. of generations.	Generation time.	Viable bacteria per 0.5 cc.	Time.	No. of generations.	Generation time.
min.		*min.*		*min.*		*min.*		*min.*
0	4,400				3,000			
120	212,000	0–120	5.5	21.8	110,000	0–120	5.1	23.5
240	2,700,000	120–240	3.6	33.3	925,000	120–240	3.07	39.0
300	5,750,000	240–300	1.09	55.0	2,125,000	240–300	1.2	50.0

Experiment 6 shows that if actively growing pneumococci are reinoculated into bouillon in which the same strain has already grown until the culture has sterilized itself (in this case after incubation at 37°C. for 4 days), they continue to multiply for several hours so that many organisms are formed. If substances are produced in a bouillon culture of pneumococcus which ultimately cause the death of many of the bacteria, then this experiment indicates that after prolonged incubation (in this case 4 days) they are no longer so potent as they were earlier, since they are not able to prevent the formation of several millions of bacteria per unit volume when an actively growing culture of the same strain is reinoculated.

Behavior of Pneumococci in Filtrates of Broth Cultures.—Experiments 4, 5, and 6 suggest that there are formed in broth cultures of pneumococcus substances which have an inhibitory or bactericidal action upon the bacteria themselves and that they lose potency when the cultures are allowed to incubate at 37°C. over a period of several days. In view of these facts, filtrates taken at different periods of growth were tested. Experiments have been reported in the literature which deal with the effect of filtrates of bacterial cultures upon the bacteria themselves when reinoculated into the filtrates. Apparently, however, the question has not been investigated from the standpoint of the relation of the age of a culture to the injurious action of its filtrate. Moreover, in previous work the cultures used for reinoculation have all been of an age sufficient to show a definite lag in the control medium, and for that reason the results are somewhat difficult of interpretation. The following experiment shows that the bactericidal action of a filtrate is dependent in part upon the age of the culture from which it is obtained.

Experiment 7.—A flask containing 1,000 cc. of bouillon was inoculated with 0.2 cc. of an 18 hour broth culture of Pneumococcus Type II, stock strain, and incubated at 37.5°C. At frequent intervals counts were made. At intervals of 1, 2, 4, and 6 days 100 cc. of the culture were removed and filtered through a Berkefeld filter. The same filter was used in each case after careful washing and sterilization. The filtrates were collected separately, and kept on ice until all were assembled and then inoculated with equal amounts of an actively growing (4 hour) culture of the same strain of pneumococcus, and incubated at 37.5°C. Bacterial counts were made at frequent intervals. The results are given in Tables X and XI.

TABLE X.

Growth of the Original Pneumococcus Type II Culture, from Which Definite Amounts Were Removed at Stated Intervals for Filtration.

Time.	Viable bacteria per 0.5 cc.	Time.	No. of generations.	Generation time.
hrs.		*hrs.*		*min.*
0	36,600			
1	34,500	0–1	0	
2	36,100	1–2	0.066	909.0
3	41,900	2–3	0.2	300.0
4	57,000	3–4	0.44	136.3
6	310,000	4–6	2.4	50.0
7	710,000	6–7	1.1	54.5
8	2,100,000	7–8	1.5	40.0
9	5,900,000	8–9	1.4	42.8
11.5	145,000,000	9–11.5	4.5	33.3
13	229,000,000	11.5–13	0.6	150.0
14	270,000,000	13–14	0.2	300.0
16	275,000,000	14–16	0.02	6,000.0
21	292,000,000	16–21	0.08	3,750.0
24*	231,000,000	21–24	0	
29.5	140,000,000	24–29.5	0	
48*	38,500,000	29.5–48	0	
75	970,000	48–75	0	
96*	2,280	75–96	0	
144*	0	96–144	0	

* At this point 100 cc. of culture were removed and filtered through a Berkefeld filter, the same filter being used in each case.

TABLE XI.

Comparison of Growth in Filtrates Obtained from a Culture of Pneumococcus Type II at Varying Periods in Its Life Cycle (See Table X).

4 hour culture of same strain of pneumococcus used for inoculation.

Time.	Viable bacteria per 0.5 cc.			
	1 day filtrate.	2 day filtrate.	4 day filtrate.	6 day filtrate.
min.				
0	1,350	1,540	1,380	1,570
60	2,300	6,250	8,800	10,600
120	4,000	11,700	28,700	44,000
180	8,500	19,800	58,000	82,000
245	8,400	28,000	82,000	127,000
330	9,000	34,000	111,000	173,000
420	8,800	46,000	169,000	225,000
515	5,000	39,000	243,000	322,000
605	3,950	35,000	330,000	254,000
695		18,000	363,000	167,000
1,350	8	570	104,000	91,000
	Clear.	Clear.	Slight cloud.	Slight cloud.

The results show that the filtrates obtained at varying intervals after the culture has passed its period of maximum rate of growth, differ in the deleterious effect which they exert upon the growth of the same strain of pneumococcus and that this inhibitory action is most marked at the time when the culture has reached the summit of its growth, after which it becomes progressively less as the culture is allowed to stand at 37.5°C., being least marked when the culture has sterilized itself.

Toxic Substances and Lag.—The experiments show that lag is a phenomenon which bacteria exhibit when the culture used for inoculation has passed the period of maximum rate of growth, and indicate that at this time substances are present in the fluid portion of a culture which exert a deleterious effect upon the bacteria, causing the death of many and temporarily inhibiting the growth of others; furthermore, that these substances are not permanent but disappear or lose their effect in part as the culture becomes older. If this interpretation of the experiments is the correct one, then, by exposing actively growing pneumococci for several hours to the action of a culture filtrate containing these deleterious substances in their maximum concentration, the bacteria should become injured and the injury should be manifested by a failure to grow at a rapid rate immediately upon inoculation into a new medium. In other words, a definite lag should be induced upon otherwise actively growing organisms by this procedure. The exposure would have to be carried out at temperatures below those at which the bacteria grow rapidly, hence in the ice box.

Experiment 8.—A 24 hour bouillon culture of Pneumococcus Type II, stock strain, was filtered through a Berkefeld filter and the filtrate found to be sterile. 1 cc. of a 6 hour broth culture of the same strain was inoculated into 50 cc. of this filtrate and a like amount inoculated into a flask containing 50 cc. of unused bouillon of the same lot as that from which the culture filtrate was obtained. The flasks were immediately placed in the ice chest for 18 hours; their contents were then centrifuged separately at high speed for 20 minutes and the supernatant fluids were removed. The sedimented bacteria were suspended in small amounts of isotonic salt solution and inoculated separately into two flasks containing 50 cc. of unused broth warmed to 37°C. The latter were incubated and the numbers of viable bacteria determined at frequent intervals. The results are given in Table XII.

TABLE XII.

Comparison of Growth of Pneumococcus Type II in Broth, after Exposure to 24 Hour Culture Filtrate and after Exposure to Unused Broth at 0°C. for 18 Hours.

Time.	After exposure to filtrate of 24 hour broth culture.				After exposure to unused broth.			
	Viable bacteria per 0.5 cc.	Time.	No. of generations.	Generation time.	Viable bacteria per 0.5 cc.	Time.	No. of generations.	Generation time.
min.		*min.*		*min.*		*min.*		*min.*
0	18,250				7,600			
60	28,200	0– 60	0.62	97.0	19,000	0– 60	1.02	58.0
110	55,000	60–110	0.93	53.0	61,000	60–110	1.6	31.5
180	170,000	110–180	1.6	43.7	312,000	110–180	2.3	30.4
240	409,000	180–240	1.2	50.0	1,070,000	180–240	1.7	35.3

Experiment 8 demonstrates that, by exposing actively growing pneumococci to the action of a culture filtrate of the same strain for a period of 18 hours at 0°C., it is possible to impose upon such bacteria a generation time almost twice as great as that shown by the control organisms. In other words, an increased lag has been induced upon such bacteria by exposing them to an environment in which they have been previously grown.

DISCUSSION.

Several views have been advanced to explain the nature of lag. The fact that bacteria, when inoculated into suitable nutritive media, do not immediately increase at the maximum rate of which they are capable, indicates that there is a lack of complete adjustment between the bacterial cell and its environment, and that this discrepancy must first be corrected before rapid growth can ensue. Obviously, one of two factors, cell or medium, must be at fault. It follows that in order that bacterium and nutritive medium may become completely adjusted to one another a change must take place in one or both of the factors. Lag, then, would represent the time necessary for such a change to be effected.

Keeping these points in mind, the possible causes of lag may be considered under two heads, according to whether the medium or

the bacterial cell is responsible. In other words, the cause may be extracellular or cellular in origin.

Extracellular Theory of the Nature of Lag.—According to this conception, the medium as such is not available for immediate growth and must first be altered by the bacteria themselves in one of the following ways: (1) by the agency of an extracellular enzyme whose function is to split up complex non-available food substances into simpler ones suitable for immediate utilization by the bacterial cell; or (2) by the elaboration of chemical substances which either exert a stimulating effect upon the metabolic processes of the bacteria (as suggested by Rahn (4)) or neutralize preformed toxic substances already present in the medium.

If the causes of lag are extracellular, then lag should always be present no matter what the age of the culture used for inoculation. That such is not the case is demonstrated by the fact that subcultures made from a parent culture when the latter is growing at a rapid rate continue to grow at the same rate and show no lag whatever. This fact, first indicated by Barber (5), later more clearly shown by Penfold (7) to be the case for colon bacilli, and further extended in our experiments with other species, disposes of the extracellular theory of lag and puts the responsibility for failure to grow upon the bacteria themselves.

Cellular Theory of the Nature of Lag.—Since bacterial lag is not due to a fault in the medium, it follows that the cause must be sought in the bacterial cell itself. In Experiments 2 and 3 it was shown that bacteria do not exhibit lag until the culture from which the inoculation has been made has ceased to grow at a maximum rate. When a culture has passed the period of maximum rate of growth the bacteria have changed in some manner since they are no longer able to multiply at once at a rapid rate when transferred to suitable medium. The nature of this change must constitute the cause of lag. The alteration which the bacteria undergo when a culture passes the period of maximum rate of growth may be explained by one or the other of the following reasons. (1) The bacterial cell may lack the proper concentration of intermediate products essential to the synthesis of bacterial protoplasm, a view suggested by Penfold (7); or (2) the bacterial cell may have sustained an injury to those functions or

parts of the cell which are concerned with the maintenance of bacterial metabolism. This conception was suggested by Müller (2) who first described the phenomenon of bacterial lag, although his experiments were not sufficient to establish his view conclusively.

The first idea is not substantiated by the facts brought out in Experiments 4 and 5 which demonstrate that when a broth culture of pneumococcus is centrifuged at the end of the period of maximum rate of growth, the bacteria in the supernatant fluid on further incubation exhibit a marked lag, whereas the bacteria in the original culture continue to grow quite rapidly. Consequently, there is a marked discrepancy in the behavior of the bacteria in the culture as a whole and of those in the supernatant fluid. Why should not the bacteria in each case behave in the same fashion? The lag which is manifested by the pneumococci in the supernatant fluid cannot be due to a lack in those organisms of sufficient intermediate products of metabolism, since the bacteria in the parent culture continue to grow rapidly for several hours at least.

When a culture is centrifuged those bacteria remaining in the supernatant fluid are exposed to the action of all the soluble substances that have been formed as a result of the growth of the culture as a whole. If such metabolic products are toxic for the bacterial cells, it would seem that their action would be stronger when the proportion of metabolic substances to bacterial cells has been increased. It would therefore seem not impossible that the marked lag which is manifested by the pneumococci under these conditions is due to an excess of deleterious substances which cause the death of large numbers and temporarily inhibit the growth of others.

The injurious substances may represent waste products from the cells or unused portions of the molecules of food substances. In any event, the amount present in the fluid is directly proportional to the number of bacteria that have been formed. For that reason they would not be present in any marked amount during the early portion of the period of maximum rate of growth and consequently the bacteria in the supernatant fluid obtained at that time would not show lag. Experiments 4 and 5 show that such is actually the case.

Additional evidence for the view that there are present in the sup-

ernatant fluid obtained at the end of the period of maximum rate of growth substances that exert an injurious effect upon the bacteria is supplied by Experiment 5, which shows that during the time that the organisms in the supernatant fluid are succumbing they do so in accordance with the law of unimolecular reactions, and in this respect their behavior is identical with that of other bacteria when subjected to the action of disinfecting agents.

Although the pneumococci in the supernatant fluid show a marked lag, many of them being killed, the fact that growth is resumed and that ultimately several millions are formed, indicates that the factors responsible for the injurious action have ceased to exist as such. This result may have been brought about through absorption of the substances by the bacterial cells, through spontaneous decomposition, or through decomposition by extracellular enzymes; or, on the other hand, through adaptation of the bacteria to the substances in question.

The idea that the substances are lost through absorption by the cells, or through decomposition or neutralization, is supported by Experiment 6, which shows that after a culture of pneumococcus has sterilized itself through incubation at 37°C. for 4 days, actively growing bacteria when reinoculated continue to grow for several hours. Additional evidence is offered by Experiment 7 which shows that culture filtrates of pneumococci manifest actual loss in inhibitory action, the longer the culture stands at incubator temperatures.

Finally, the view that broth cultures of pneumococcus contain substances that have the property of exerting a direct deleterious effect upon the bacteria themselves is supported by Experiment 8, which demonstrates that it is possible to impose upon actively growing pneumococci an increased generation time by exposing them at ice box temperatures to the action of a filtrate of a 24 hour broth culture of the same strain.

To sum up: (1) Bacterial lag represents an alteration of the bacterial cell. (2) The alteration is concerned with that function or structure of the cell which is essential to metabolism and hence growth of the cell, and is to be regarded in the nature of an injury. (3) The injury is due to the exposure of the cell to direct or indirect products of its own metabolism. (4) The injury may be sufficient

to bring about the death of the cell or it may merely inhibit growth temporarily. (5) The alteration is not always permanent. The bacteria may overcome or survive the condition, or the agents responsible for producing it may suffer actual destruction or be rendered inert. (6) Inasmuch as different media vary in their suitability for bacterial growth, the injury is likely to be most marked when bacteria are grown in a medium which is least favorable.

Stated briefly, it is supposed that if bacteria upon inoculation into suitable media do not begin to multiply immediately at the maximum rate of which they are capable, it is because they have sustained an injury from their former environment.

This view is in accord with the facts elicited by the experiments reported in this paper. It is essentially the view suggested by Müller, although, as already stated, his experimental evidence was meager. It was also advanced by Barber who thought he could distinguish differences in the morphology of the individuals of a 24 hour culture of colon bacilli and those of a culture that was growing rapidly.

The work of Moore (13) lends strong support to this conception of the nature of lag. He has shown that the blood serum of rabbits that have received certain amounts of ethylhydrocuprein (optochin) acquires the property of destroying pneumococci *in vitro*. If smaller amounts of the drug be administered, however, the serum is no longer able to kill the pneumococci, but increases the duration of lag.

The idea that bacteria form toxic substances which in turn inhibit the growth of the organisms is of course not a new one and has given rise to two opposing schools of opinion (14). The evidence here presented indicates that such substances are formed, and that they exert a direct injurious effect upon the microorganisms. Furthermore, these substances appear much earlier than is generally supposed, and on this account it would seem necessary to obtain accurate knowledge of the growth curve of a culture before the terms "old" or "young" are applied to it. Failure to do so has in the past led to erroneous conclusions as to the nature of lag. What is most important to recognize is the fact that the members of a bacterial culture vary considerably in their ability to grow in a given medium, depending upon the particular phase in which the original culture happens to be at the time of transplantation. The bearing

of this fact upon the possibility of variations in virulence of the members of a culture at different stages in its growth is at present under investigation.

Inasmuch as the bacteria in a culture which has passed its period of maximum rate of growth seem to have sustained an injury, it is suggested that in testing the efficacy of any bactericidal agent, actively growing cultures should be used, for if organisms already injured are employed, the result would be a summation of effects due to the agent and those represented by the injury to the bacteria, hence exaggerated ideas might be derived concerning the efficacy of the agent tested. Evidence in favor of this view is offered by the work of Chick (12) who has shown that the individuals of a 3 hour culture of *Bacillus paratyphosus* are more resistant to the action of antiseptics than those of an 18 hour culture of the same strain.

In order to preserve cultures over a long period of time, it has been found best to put on ice those which are in the period of maximum rate of growth, indicated in the case of broth cultures of pneumococcus by the presence of slight turbidity. Such bacteria retain their viability over a longer period than those of 18 hour cultures, hence necessitate less frequent transfers and give greater assurance of recovery, a factor of no little importance in the care of large collections of cultures.

CONCLUSIONS.

1. Cultures of *Diplococcus pneumoniæ*, *Bacillus coli*, *Bacillus fluorescens liquefaciens* and *Bacillus prodigiosus*, when grown in meat infusion broth exhibit an initial latent period when the culture used for inoculation is no longer growing at its maximum rate; if, however, the culture is growing at its most rapid rate the bacteria, upon subculture, show no latent period but continue to multiply at the same rate as that of the parent culture.

2. If broth cultures of pneumococcus are centrifuged at the beginning of the period of maximum rate of growth, the bacteria remaining in the supernatant fluid continue to grow at a rapid rate upon further incubation; if, however, the culture is centrifuged at the end of the period of maximum rate of growth, those bacteria which remain in the supernatant fluid show a prolonged latent period, during

which many of the organisms die. While the death of these bacteria is taking place the process follows closely the law of unimolecular reactions.

3. Actively growing pneumococci inoculated into the supernatant fluid from a 4 day culture of the same strain continue to grow rapidly for an appreciable time after inoculation.

4. Filtrates from 24 hour cultures of pneumococcus inhibit the further growth of actively growing pneumococci when the latter are inoculated into such filtrates. This inhibitory action of the filtrates is lost in part as the culture from which the filtrate is obtained is allowed to incubate longer.

5. Actively growing pneumococci, after exposure at low temperatures to the action of the filtrate of a 24 hour broth culture of the same strain, show a greater lag than the controls.

6. The foregoing facts offer strong support for the view that lag is an expression of injury which the bacterial cell has sustained from its previous environment.

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A RAPID METHOD FOR THE DIAGNOSIS OF RENAL TUBERCULOSIS BY THE USE OF THE X-RAYED GUINEA PIG.

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The value of animal inoculation in the diagnosis of tuberculosis has been recognized since Marcet's[1] experiments in 1867. Klebs[2] confirmed this work in the following year, and the constant employment of the guinea pig test since that time has proved it to be the safest diagnostic method that we have.

It is natural that this method should be given special importance in cases of renal tuberculosis where there is always the complicating smegma bacillus to rule out on microscopic examination, and where the demonstration of the tubercle bacillus is a matter of considerable difficulty. In the past year Crabtree[3] published his technique for high centrifugalization of the urine, an important step forward, but in the same article he admits that guinea pig inoculation must be the final test in all doubtful cases.

Damsche[4] was the first investigator to use the guinea pig for the diagnosis of renal tuberculosis. From his time to the present there has been little change in the technique employed. In the Massachusetts General Hospital a subcutaneous injection of 15 to 20 minims of suspected urine is made into the abdominal wall of the animal and after 5 weeks an autopsy is done. The diagnosis is made both by gross lesions and by microscopic examination of film preparations. Barney and Young's[5] summary of 197 cases from this hospital shows

[1] Marcet, W., *Tr. Med. Clin.*, 1867, 437.

[2] Klebs, E., *Virchows Arch. path. Anat.*, 1868, xliv, 242, 296.

[3] Crabtree, E. G., *Surg., Gyn. and Obst.*, 1916, xxii, 221.

[4] Damsche, *Deutsch. Arch. klin. Med.*, 1882, xxxi, 78 ff.

[5] Barney, J. D., and Young, E. L., Jr., *Boston Med. and Surg. J.*, 1911, clxiv, 917.

that the reports previous to operation have been correct in 99.5 per cent of the cases.

The method is, however, open to criticism on account of the time that is lost while waiting for the lesions to develop in animals.[6] Any modification, therefore, that will allow us to shorten the time appreciably should be of practical value. With this in mind, the following experiments were undertaken.

Murphy and Ellis[7] have shown that white mice which have been exposed to x-rays are made markedly more susceptible to bovine tuberculosis than normal animals. They explain this fact by the destruction of the lymphoid tissue which they claim constitutes an important agent in the defensive mechanism against tuberculosis. It seemed reasonable to assume that the guinea pig could be rendered less resistant than it is normally by a similar procedure. Accordingly, guinea pigs were given various doses of Roentgen rays to determine how much they could stand and whether the resistance to known tuberculous urine could be reduced.

It was found that guinea pigs could tolerate a large amount of x-rays without apparent injury to their health. The average normal white blood count of the animals was from 12,000 to 15,000 cells per c. mm., of which 40 to 45 per cent were of the lymphocytic variety, and, with the exception of $\frac{1}{2}$ to 1 per cent of transitionals and large mononuclears combined, the remaining cells were polymorphonuclears. One massive treatment with x-ray sufficed to reduce the total count by over one-half (4,000 to 6,000), mainly effected by reduction in the lymphoid cells. The count remained depressed for a period of over 1 week, which was as far as it was followed. Animals treated in this way when inoculated with a known tuberculous urine developed marked lesions of the disease in a much shorter time than did normal animals. Further experiments showed that an x-ray exposure given shortly after inoculation was as effective in reducing the resistance as when given before the inoculation. This fact seems to be of practical importance as it obviates the necessity of keeping a stock of treated animals on hand. After having established a working dosage and the best time interval before killing the animals, a series of cases was studied in connection with the Genito-Urinary

[6] Churchman, J. W., *Med. Rec.*, 1916, lxxxix, 511.

[7] Murphy, Jas. B., and Ellis, A. W. M., *J. Exp. Med.*, 1914, xx, 397.

Department of the Massachusetts General Hospital, in order to test the usefulness of the method.

It was considered necessary for practical reasons to confine the x-ray preparation to one exposure only, sufficiently massive to carry the animals through 10 days with depressed resistance. It is probable, however, as is shown by the experiments of Murphy and Morton,[8] that repeated small exposures would be more satisfactory for experimental study when it is desirable to produce a maximum effect on the lymphoid tissue with a minimum damage to other structures. The following dosage was adopted as the most useful. The animal was placed in a pasteboard box of such a size that it could not move around, and then radiated for a period of 10 minutes with the Coolidge tube, the target being 12 inches distant from the base of the box. A 5 milliampere current was passed through the tube, backing up $8\frac{1}{2}$ inches of spark between points. The Snook interrupterless was the type of machine employed. No aluminum or leather filters were used. From 1 to 2 cc. of urine were injected intraperitoneally according to the amount at hand. After 10 days the guinea pigs were killed and examined for the lesions of tuberculosis. As in the normal animals, the usual sites of election were the spleen, mesenteric lymph glands, and liver. The lesions varied with the severity of the infection,—sometimes the spleen was riddled with a mass of caseous nodules 2 to 4 mm. in diameter, while at other times the spots were more nearly the size of a pinhead but distinctly pathognomonic. The lymph glands were usually from 1 to 1.5 cm. in diameter and caseous, and, as a rule, the liver had scattered over its surface nodes similar in size to those of the spleen. Films taken from these situations and stained for tubercle bacilli were used to confirm the gross diagnosis. Microscopically the picture was one of coagulation necrosis and caseation with a zone of endothelioid cells but no formation of giant cells, and a striking absence of the small round cell infiltration. There was also no evidence of an enclosing fibrous layer such as is commonly seen in the slow growing tubercle.

The following cases were taken at different times from the Genito-Urinary Department, and the above method of making the diagnosis was used. The guinea pig findings were confirmed in every case as far as possible by all other data which would tend to throw light on possible error. The results are given in Table I.

[8] Murphy, Jas. B., and Morton, J. J., *J. Exp. Med.*, 1915, xxii, 204.

TABLE I.

Case No.	Urine.	Cystoscopy.	Normal guinea pig test.	X-rayed guinea pig test.	Operation.	Pathological report.	Clinical diagnosis.	Remarks.
1	Large numbers of tubercle bacilli in clumps. Culture not done.	Tuberculous bladder.	1. Killed in 5 weeks. Spleen full of nodules of tubercle bacilli. 2. Died during night.	Both animals died on following day.	None.	None.	Tuberculosis of both kidneys and of lungs.	One normal and two x-rayed animals died in 24 hrs. from a mixed streptococcus infection. One normal animal showed tuberculous lesions of spleen after 5 weeks.
2	Sediment, from left kidney showed tubercle bacilli; from right kidney few tubercle bacilli. Culture, no growth.	Tuberculous pus in left ureter. No pus in right ureter.	No test.	Killed on 10th day. Liver covered with patches. Spleen contained small nodules. Glands caseous.	"	"	Tuberculosis of left kidney. Tuberculosis of right kidney? Tuberculosis of lungs.	Question of right kidney excreting bacilli without disease. No pus in urine on right side. Tubercle bacilli in sputum.
3	Sediment, from left kidney showed few tubercle bacilli; from right showed tubercle bacilli. Culture, few cocci from left; no growth from right.	"Not diagnostic."	Not yet reported.	Killed on 7th day. Extensive lesions in liver, spleen, and glands.	"	"	Tuberculosis of both kidneys.	
4	Sediment, many tubercle bacilli. Culture not made.	Not done.	No test.	Extensive lesions in liver, spleen, and lymph glands.	Nephrectomy.	Tuberculosis of kidney.	Tuberculosis of kidney.	

5	Sediment, few tubercle bacilli after high centrifuge. Culture, no growth.	Could not catheterize.	"	Killed on 10th day. Spleen riddled. Glands caseous; size of 10 cent piece.	"	"	Tuberculosis of kidney or bladder.	
6	Sediment, negative for tubercle bacilli. Culture, no growth.	Nothing suggestive.	Positive for tubercle bacilli in 5 weeks.	Killed on 9th day. Spleen and lymphoid glands caseous.	"	"	Tuberculosis of kidney.	Two x-rayed guinea pigs used. First killed on 9th day with diagnostic lesions; second killed in 5th week with severe lesions.
7	Sediment, negative for tubercle bacilli. Culture, no growth.	Left ureter retracted. Tuberculosis?	Negative four times.	Killed on 8th day. Liver peppered with pin-point lesions. Spleen also involved.	"	"	Tuberculosis of kidney.	Four normal guinea pigs gave negative results.
8	Sediment, colon bacilli. Culture not made.	Ulcer near right ureter, tuberculous.	No test.	Killed on 14th day. Liver and spleen contained pin-point caseous nodules.	"	"	Tuberculosis of right kidney.	
9	Sediment, no bacilli. Culture not made.	Suggests bilateral tuberculosis.	"	Died on 8th day. Peritonitis. Film showed tubercle bacilli present.	"	"	Tuberculosis of left kidney. Pott's spine. General peritonitis.	Mixed infection with doubtful lesions.
10	Sediment, negative for tubercle bacilli. Culture, streptococci.	Not done.	"	Killed on 14th day. Marked lesions in liver. Spleen riddled. Glands caseous.	None.	None.	Tuberculous epididymitis. Tuberculosis of bladder or kidney?	

TABLE II.

Case No.	Urine.	Cystoscopy.	Normal guinea pig test.	X-rayed guinea pig test.	Operation.	Pathological report.	Clinical diagnosis.	Remarks.
1	Sediment, no tubercle bacilli. Culture, staphylococci.	Normal.	Negative for tubercle bacilli.	Killed on 14th day. Marked lesions in liver and spleen.	None.	None.	Pyonephrosis or pyelitis.	Film cleared diagnosis. Cocci. Negative for tubercle bacilli.
2	Sediment, no tubercle bacilli; colon bacilli. Culture, colon bacilli.	"Bladder looks like subsiding colon infection."	No test.	Killed on 12th day. Plastic peritonitis. No tuberculosis.	"	"	Cystitis.	
3	Sediment, no tubercle bacilli. Culture, staphylococci.	"Practically normal."	Negative for tubercle bacilli.	Killed on 10th day. No lesions.	"	"	"	
4	Sediment, no tubercle bacilli. Culture, staphylococci.	Impossible.	"	Killed on 10th day. No lesions.	"	"	Cystitis. Renal stone.	
5	Sediment, no tubercle bacilli. Culture, staphylococci.	Not done.	"	Killed on 10th day. General peritonitis.	"	"	Cystitis.	
6	Sediment, no tubercle bacilli. Culture, colon bacilli.	"Suggestive of tuberculosis."	"	Killed on 10th day. Foul general peritonitis. Culture, staphylococci.	"	"	Pyonephrosis. Cystitis.	

7	Sediment, no tubercle bacilli. Culture, colon bacilli.	Not done.	"	Killed on 10th day. Urine from left and right ureters negative.	"	"	Cystitis.	
8	Sediment, no tubercle bacilli. Culture, staphylococci.	"	No test.	Died on 7th day. General peritonitis. No tuberculosis.	"	"	"	
9	Sediment, no tubercle bacilli. Culture, streptococci.	Irritable bladder, small capacity. Could not catheterize ureter.	"	Killed on 10th day. Bladder urine gave no lesions.	"	"	"	
10	Sediment, left, few cocci; right, no tubercle bacilli. Culture, left, staphylococci; right, no growth.	Normal.	Negative for tubercle bacilli.	Killed on 10th day. Urine from left ureter gave small lesions. Film, cocci. Urine from right ureter negative.	"	"	Pyelitis.	Film rules out atypical lesions. No tubercle bacilli.

Cases 1, 2, and 3 were extensive renal tuberculosis, bilateral, and of course inoperable. They were used early in the series and are included to make the gradations of severity complete. Case 1 is instructive in that the x-rayed guinea pigs and one of the normal animals were killed by a mixed streptococcus infection. The other normal guinea pig survived, and when it was killed after 5 weeks, the spleen was full of tuberculous nodules. Case 2 presents an interesting possibility; namely, that the left kidney alone was involved, and the right was excreting tubercle bacilli without being affected. That this does occur at times is recognized in the literature (Brown[9]). Cases 4 and 5 were moderately severe and the bacilli were found in the urine by use of the high centrifuge method. Cases 6 to 10 were not advanced cases and no tubercle bacilli were demonstrated by microscopic examination of the sedimented specimens. Case 7 was tried four times in normal guinea pigs with negative results, probably because the ureter was occluded. On the first trial with the x-rayed guinea pig the test was successful in 8 days, an example of good fortune in getting a specimen which contained the bacilli. Case 9 had a mixed infection and the lesions were atypical. Acid-fast bacilli were demonstrated in films made from the animal, but the report would not have been made as positive without repetition except for the evidence of Pott's disease of the spine in this case. Most of these cases were reported within the 10 day limit, but three of the earlier ones were allowed to go 2 weeks before a diagnosis was made. These three cases would have undoubtedly fallen into the same period as the others, as the lesions were extensive in each instance.

A table of the non-tuberculous conditions in which it was necessary to rule out tuberculosis is also presented (Table II).

In these ten cases the diagnosis "negative for tuberculosis" has been confirmed by the clinical course, by tests with normal guinea pigs, and by all other methods available. Cases 1 and 10 (same individual) were the only ones which gave lesions in any way comparable to tuberculosis. In these cases there were multiple abscesses which in films showed no tubercle bacilli, but many small micrococci. Other guinea pigs in the series died early of plastic peritonitis from which pure cultures of the organism responsible were recovered.

[9] Brown, L., *J. Am. Med. Assn.*, 1915, lxiv, 886.

DISCUSSION.

While the number of cases here presented is comparatively small, the uniformity of the results justifies a report at this time. The work is of value from the experimental side in that it confirms and extends the observation of Murphy and Ellis, in which they showed that white mice were made markedly more susceptible to bovine tuberculosis by suitable x-ray exposures. These experiments demonstrate that the same conditions hold for the human strain of the organism in the guinea pig. As to whether this lowered resistance is due to the destruction of the lymphoid elements as is suggested by Murphy and Ellis, the present work is perhaps not conclusive. For a demonstration of this point it would be better to use the fractional dose method as recommended by Murphy where a maximum effect may be obtained on the lymphoid elements with a minimum on the other structures. For obvious reasons, however, this method would not be practical for clinical use.

SUMMARY.

It is possible to reduce the resistance of guinea pigs to tuberculosis by x-ray exposures, so that when animal inoculation is required for a diagnosis a much quicker result may be had than by the use of normal animals. In renal tuberculosis when it is necessary to resort to the use of animals, it ordinarily requires from 5 to 7 weeks, while by the use of x-rayed guinea pigs the diagnosis can be made in from 8 to 10 days. The resistance can be sufficiently lowered by one massive dose of x-ray administered either shortly before or after the inoculation of the material to be tested. The lesions are so marked in these animals that the diagnosis is certain after the interval indicated above.

I wish to thank the members of the Staff of the Genito-Urinary Department for the interest they have taken and the assistance they have rendered in making this work possible.